HDL Mimetic Peptides as Potential Therapeutics for Alzheimer's Disease

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Dedication

This dissertation is dedicated to my grandfather, Dr. Harry Chernick, who passed after a prolonged battle with Alzheimer's disease in 2007. He was a brilliant man, and kind, and the memory of his laughter will continue to motivate my efforts to improve outcomes for dementia patients for the remainder of my life.

This dissertation is also dedicated to my grandmother, Janet Kuhn, who is currently battling with vascular dementia. Her ready smile and loving energy have encouraged my research efforts in the pursuit of a remedy to this terrible disease.

Abstract

Alzheimer's disease (AD) is the leading cause of dementia worldwide, for which there currently exists no approved disease modifying treatment. A number of large scale human clinical studies have confirmed a robust connection between high density lipoprotein (HDL) – known as the 'good cholesterol' levels and AD. Low levels of HDL are associated with increased risk and severity of AD. The role of HDL in the brain is not fully established, however, the anti-inflammatory and anti-oxidative properties of HDL are thought to be critical for its beneficial effects. Apolipoprotein E (apoE) is a key constituent of HDL-like particles in the interstitial fluid (ISF) and cerebral spinal fluid (CSF) in the brain. ApoE exists in 3 common variants in the human population (apoE2, E3, and E4), and the apoE4 isoform is the strongest genetic risk factor for AD, accounting for 40-60% of cases. This risk allele is known to increase neuroinflammation and to promote the aggregation and deposition of amyloid beta (Aβ) in the brain, effects which are influenced by the poor lipidation status of apoE4 (incomplete or improper composition of HDL-like particles) in the brain. Previous studies in the laboratory of Dr. Ling Li have shown that overexpression of human apoA-I, the primary apolipoprotein associated with HDL in the periphery, mitigated amyloid pathology and rescued memory deficits in AD mice. However, a full-length, glycosylated protein is extremely difficult and costly to synthesize and to administer. Therefore, the goal of my research was to test the therapeutic potential of small HDL-mimetic peptides, designed to mimic the beneficial function of their parent apolipoproteins, in AD. My studies focused on 4F, an 18 amino acid HDL-mimetic peptide that has been shown to be safe and well tolerated in human clinical trials for cardiovascular disease. I have demonstrated that the lipidation state of apoE is negatively impacted by the addition of aggregated Aβ to astrocytes from mice and humans, *in vitro*, an effect that is reversed by the addition of 4F. In addition, I

confirmed that apoE4 is less lipidated than apoE2 and E3 at baseline, and demonstrated that apoE4 is more susceptible to the detrimental effects of Aβ on lipidation than apoE2. Intriguingly, 4F was able to completely rescue this effect, bringing apoE4 lipidation levels on par with those of apoE2, even in the presence of Aβ. Preliminary *in vivo* studies in mice expressing the human apoE isoforms and in a mouse model of AD indicate that 4F reduces soluble amyloid levels in the brain and attenuates memory deficits. As chronic neuroinflammation is a key hallmark of AD pathology, another line of my research focused on a small molecule, called Minnelide. Minnelide is a water soluble, pro-drug of triptolide, which is an anti-inflammatory agent that has been shown in Dr. Li's lab and in other labs to mitigate AD pathology and rescue memory deficits in animal models. Poor solubility hinders this agent's prospects in the clinic, and so we sought to test the efficacy of Minnelide in AD. My studies show that Minnelide attenuated age-related cognitive decline in AD mice, independent of Aβ levels in the brains of these animals. These data, taken together, indicate that HDL mimetic peptides, and targeting of inflammatory pathways in the periphery and in the brain are promising avenues for continued efforts to find an effective treatment for AD.

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List of Abbreviations

ABCA1, ATP-binding cassette type A1

ABCA7, ATP-binding cassette type A7

ABCG1, ATP-binding cassette type G1

ActD, Actinomycin D

AD, Alzheimer's disease

ADAM10, A disintegrin and metalloproteinase 10

AKT, Protein kinase B

ANOVA, Analysis of variance

apo, Apolipoprotein

apoA-I, Apolipoprotein A-1

apoE, Apolipoprotein E

apoE2, Apolipoprotein E2

apoE3, Apolipoprotein E3

apoE4, Apolipoprotein E4

apoJ, Apolipoprotein J

APP, Amyloid precursor protein

Aβ, Amyloid-β peptide

BACE1, Beta-secretase 1

BBB, Blood-brain barrier

BFA, Brefeldin A

C3, Complement component 3

CAA, Cerebral amyloid angiopathy

CE, Cholesterol esters

CETP, Cholesteryl ester transfer protein

CHX, Cycloheximide

CLU, Clusterin or apoJ

CNS, Central nervous system

CSF, Cerebrospinal fluid

CVD, Cardiovascular disease

DAPI, 4',6- diamidino-2-phenylindole

DMEM, Dulbecco's Modified Eagle Medium

EAE, Experimental autoimmune encephalomyelitis

EGF, Epidermal growth factor

ELISA, Enzyme-linked immunosorbent assay

ER, Endoplasmic reticulum

ER, Extended release

ERK, Extracellular signal-regulated kinase

FC, Unesterified free cholesterol

FITC, Fluorescein isothiocyanate;

GFAP, Glial fibrillary acidic protein

GWAS, Genome-wide association studies

HDL-C, High-density lipoprotein cholesterol

HDL, High-density lipoprotein

HDR, Homology directed repair

Hep, Heparinase I

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

KO, Knock out

LCAT, Lecithin cholesterol acyltransferase

LDL, Low density lipoprotein

LDLR, Low density lipoprotein receptor

LPS, Lipopolysaccharide

LRP1, Low density lipoprotein receptor-related protein 1

LTP, Long-term potentiation

LXR, Liver X receptor

MAPK, Mitogen-activated protein kinases

MCI, Mild cognitive impairment

MFI, Mean fluorescence intensity

MI, Myocardial infarction

miRNAs, MicroRNAs

Mo, Months old

mRNA, Messenger RNA

nAChR, Nicotinic acetylcholine receptor

NDGGE, Non-denaturing gradient gel electrophoresis

NGF, Nerve growth factor

NMDA, N-methyl D-aspartate

nTg, Non-transgenic

P-tau, Phosphorylated tau

PBS, phosphate buffered saline

PDL, Poly-D-Lysine

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

PL, Phospholipids

PLTP, Phospholipid transfer protein

Pro, Pronase E

PS1, Presenilin-1

RCT, Reverse cholesterol transport

RFP, Red fluorescent protein

RNA, Ribonucleic acid

rHDL, Recombinant high-density lipoprotein

RXR, Retinoid X receptors

SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SE, Standard error

SNP, Single nucleotide polymorphism

SR-B1, Scavenge receptor B1

SwDI, Swedish Dutch and Iowa mutations of APP

TEM, Transmission electron microscopy

Tg, Transgenic

TGs, Triglycerides

TR, Targeted replacement

TREM2, Triggering receptor expressed on myeloid cells 2

TxRed, Texas Red

Veh, Vehicle

VLDL, Very low-density lipoprotein

WT, Wild type

CHAPTER 1 – LITERATURE REVIEW

Overview of Alzheimer's Disease

Alzheimer's disease demographics

Alzheimer's disease (AD) is a progressive age-related neurodegenerative disorder, which leads to cognitive impairment and ultimately death. AD is the most common cause of dementia in the elderly, and currently affects over 5 million Americans, nearly two-thirds of whom are women (Alzheimer's Association, 2018). This number is expected to rise to over 15 million by the year 2050, as our population continues to age. Mild cognitive impairment (MCI), a condition that often leads to full-blown AD, affects approximately 20% of individuals over the age of 65 (Alzheimer's Association, 2018).

Compared with many other major diseases, treatment for AD has remained stagnant, as there remains no available therapeutic to stop the progression of this devastating disease. This is starkly illustrated when looking at disease-related deaths in recent years: HIV-related deaths are down 55% since the year 2000, while AD-related deaths are up more than 120% in the same time-frame (Alzheimer's Association, 2018).

The cost of care for AD patients can be crippling for families, and devastating for society. The average cumulative lifetime cost of care for an AD patient has been estimated at nearly $350,000, with 11.4 billion dollars in health care costs, each year, across America (Alzheimer's Association, 2018). This does not even begin to account for lost wages, or the emotional and physical strain AD places on patients, caregivers, and loved ones. Family members are often caregivers for AD patients, which can take an enormous toll mentally, while also pulling people away from work and other duties (National Alliance

for Caregiving, 2017). Alarmingly, non-professional caregiving has been associated with increased risk of mortality as well (Roth et al., 2015).

Pathological hallmarks of Alzheimer's disease

The pathogenesis of AD, particularly the sporadic form of AD, is not fully understood. However, significant experimental and clinical efforts over the past decades has led to a robust understanding of many environmental and genetic risk factors, and relevant pathways. The defining pathological hallmarks of the AD brain, first described by Dr. Alois Alzheimer over 100 years ago, include intracellular neurofibrillary tangles of tau protein and extracellular deposits of amyloid-β protein (Aβ) in neuritic plaques within the brain parenchyma. Great strides have been made in understanding the contributions of Aβ and tau to AD pathogenesis and clinical manifestation in recent years.

There are two forms of AD, familial and sporadic, which differ in their age-at-onset and causal factors (Mayeux and Stern, 2012). Familial AD, which afflicts only 1-5% of all AD cases (Alzheimer's Association, 2018), is caused by mutations that result in elevated production of Aβ in the brain, and can be passed down to offspring (Waring and Rosenberg, 2008). Compared with familial AD, sporadic AD, also known as late-onset AD, occurs at a later age (average age at onset; 55 years of age for familial AD, 75 years of age for sporadic AD) (Koedam et al., 2010). The casual mechanisms of sporadic AD are not as well understood, although the accumulation of Aβ is thought to play a pivotal role in this form of the disease as well (Hardy and Selkoe, 2002).

Aβ is a small peptide (typically 37-43 amino acids in length), derived by sequential cleavage of the transmembrane protein, amyloid precursor protein (APP), by β- and γ-secretase (Thinakaran and Koo, 2008; Haass et al, 2012). APP plays important roles in promoting neuronal viability and synaptic activity, and it remains unclear to what extent

AD is driven not only by generation of Aβ, but also by loss of normal APP function (Perez et al., 1997; Müller et al., 2012). Similarly, recent studies have suggested physiologic roles for Aβ as well, including modulation of cholinergic signaling, and protection against infection (Kumar et al., 2016a; Kumar et al., 2016b).

The amyloid hypothesis, which has driven much of the research in the field of AD in the past decades, posits that overproduction, failed clearance, and aggregation of Aβ are the causal and driving factor in sporadic AD pathogenesis (Hardy and Selkoe, 2002). It is important to note that other hypotheses have also been suggested (Morgen et al., 2015), and a long string of failed anti-amyloid clinical trials has resulted in some researchers questioning the validity of the amyloid hypothesis (Kametani et al, 2018). Nevertheless, the significant body of evidence supporting a direct role of Aβ in neurotoxicity and cognitive decline in cells, animals, and humans is difficult to refute, and many ongoing trials continue to target this pathway, albeit with earlier intervention and improved study design as a key focus (Kumar et al, 2016a).

The pathogenic aggregation and accumulation of Aβ in the cortex and hippocampus occurs due to overproduction and/or failed clearance mechanisms in aging and AD (Selkoe, 2001; Tanzi et al., 2004; Mawuenyega et al., 2010; Patterson et al., 2015). This buildup of Aβ is an early event in AD, preceding tau pathology and cognitive impairment (Mann et al., 1988; Jack et al., 2010). Amyloid deposition within the brain does not constitute a diagnosis of AD, as patients with other dementias often also have Aβ deposits (Bibl et al., 2006), and many cognitively normal individuals have accumulation of the peptide in their brains as well (Jagust, 2016). It is important to note, however, that given the role of Aβ in early AD pathogenesis, healthy individuals with amyloid deposition may simply be pre-symptomatic or undiagnosed (Ten Kate et al., 2018).

Importantly, amyloid fibrils are also found deposited in the cerebral vasculature, a phenomenon known as cerebral amyloid angiopathy (CAA), which reduces cerebral blood flow and disrupts the blood-brain barrier (BBB) (Olichney et al., 2000; Bell and Zlokovic, 2009). CAA is observed in approximately 80% of AD patients, and these vascular deposits are comprised primarily of $A\beta_{40}$, in contrast to plaques in the brain parenchyma, which more commonly contain $A\beta_{42}$ (Serrano-Pozo et al., 2011).

Tau protein has been thought to function normally in stabilizing microtubules, a critical role in supporting axonal health and activity, although a recent report indicates that tau may enable the elongation of microtubule labile domains (Qiang et al., 2018). However, the pathological hyperphosphorylation and aggregation of tau leads to the development of intracellular neurofibrillary tangles, a key hallmark of AD (Grundke-Iqbal et al., 1986; Wood et al., 1986). Elevated levels of tau in the CSF and in the brain are associated with conversion from MCI to AD, faster cognitive decline, and increased risk of mortality (Blom et al., 2009; Wallin et al., 2009; Sämgård et al., 2010). In line with the amyloid hypothesis, tau pathology occurs after amyloid accumulation (Gómez-Isla et al., 1997; Iqbal and Grundke-Iqbal, 2002), and precedes neuronal damage and cognitive decline.[43]

Neuroinflammation, characterized by elevated levels of proinflammatory cytokines and the activation of glial cells, is a key pathological hallmark of AD (Wyss-Coray, 2006, Heneka et al., 2015), and a major focus of the present dissertation. Both Aβ and tau have been shown to directly contribute to neuroinflammation (Sondag et al., 2009; Khandelwal et al., 2012), and other important genes related to AD risk, including apolipoprotein E (apoE), mediate their effects in part through inflammatory pathways (Tai et al., 2015). Glial activation is thought to be an early pathogenic event in AD, occurring prior to the onset of tau pathology (Parbo et al., 2018), and neuroinflammation

is itself thought to promote AD pathology as well (Zhang et al., 2013), creating a feed-forward cycle.

FDA-approved agents and difficulty in Alzheimer's disease clinical trials

To date there exists no effective disease-modifying therapy to reverse, halt, or even slow progression of AD. Five agents have been approved for the symptomatic treatment of AD by the FD, one of which was discontinued. The available therapeutics operate by one of two mechanisms, either inhibition of acetylcholinesterase or antagonism of NMDAR. The acetylcholinesterase inhibitors include donepezil, galantamine, rivastigmine, and tacrine, which was discontinued in 2013 due to safety concerns.

The relevant mechanism of these acetylcholinesterase inhibitors is thought to be preservation of the neurotransmitter acetylcholine in the brains of AD patients, which is depleted over time as neurons producing the transmitter atrophy due to AD pathology (Raskind et al., 2000; Burns et al., 1999; Camps and Muñoz-Torrero, 2002; Whitehouse et al., 1981, 1982; Wong et al., 1999). By blocking the degradation of acetylcholine, these agents counteract the loss of signalling from dying neurons. Unfortunately, as these agents do not impact the underlying pathology, neurons continue to perish and the treatments lose efficacy over time (Sun et al., 2008). The lone NMDA receptor antagonist, memantine, works to reduce glutamate excitotoxicity that is prevalent in patients with moderate-to-severe AD (Rogawski and Wenk, 2003). Inhibition of this over-stimulated pathway delays the onset of hallucinations and other common AD-related symptoms. Unfortunately, the efficacy of memantine is lower than originally thought (Gauthier et al., 2008; Winblad et al., 2007) although extended release (ER) formulations have been developed to improve the pharmacokinetic profile (Plosker, 2015). ER

formulations of the other approved agents have also been developed (Seltzer, 2010; Gadiko et al., 2013; Frampton, 2014).

Ongoing efforts

The most recently approved of the available agents, galantamine, was first approved by the FDA in 2004. In over a decade, clinical trial results have been largely disappointing across a wide array of highly anticipated agents. Chiefly, anti-amyloid agents have demonstrated efficacy in lowering Aβ levels in the brain, however, improvements in cognitive tests has been more difficult to demonstrate (van Dyck, 2018). Epidemiological evidence suggests a protective effect of anti-inflammatory agents, including NSAIDs and statins, however, clinical trials of these agents failed to demonstrate improvement in cognition in AD patients (Aisen et al., 2000; Simmons et al., 2002; Reines et al., 2004; Feldman et al., 2010). Antibody-based therapeutics, one of the most prevalent and anticipated classes of anti-AD agents, have been developed to target Aβ, tau, BACE1, apoE (Lambracht-Washington et al., 2013; Vassar, 2014; West et al., 2017; Liao et al., 2018). These agents have demonstrated significant improvement in AD pathology in animal models, yet clinical trials have been disappointing thus far (Egan et al., 2018; van Dyck, 2018). Despite this, immunotherapies continue to make up a large portion of recruiting and ongoing AD clinical trials in 2018 (Cumming et al., 2018). It is widely believed that earlier intervention and more selective inclusion criteria may be required for disease modifying therapies to reach their primary endpoints in future clinical trials designed to treat AD. In line with this hypothesis, a recent phase 2 study of 856 patients with MCI or mild AD showed that 18 months of treatment with BAN2401, an antibody directed against protofibrillar forms of Aβ, reduced amyloid burden and improved cognitive scores as early as 6 months into treatment (http://investors.biogen.com/news-releases/news-release-details/eisai-and-biogen-announce-positive-topline-results-final).

Further, the most recent update from the ongoing open-label extension trial of aducanumab, an anti-Aβ antibody that targets aggregated forms of the protein, showed reduced amyloid by PET imaging and improvement in cognitive scores, although side effects remain a concern (https://www.alzforum.org/therapeutics/aducanumab). The discovery and validation of plasma, CSF, and brain imaging biomarkers has come a long way in the past decade, and continued efforts will further improve our ability to design clinical trials with better inclusion criteria and monitoring of primary and secondary outcomes (Sutphen et al., 2014). Hopefully, these efforts will soon lead to the first disease-modifying agent approved for AD treatment.

Mouse models of Alzheimer's disease

There have been many efforts to create transgenic animal models of AD to recapitulate pathological hallmarks and cognitive deficits of human disease. The vast majority of these model systems have been developed in mice, although some rat models exist Benedikz et al., 2009). Many animal models utilize overexpression of familial mutations in APP, which lead to elevated levels and deposition of Aβ in the form of neuritic plaques, similar to those observed in human AD brains at autopsy (Holtzman, 2007). However, these models typically fail to recreate other key pathological hallmarks of AD, namely, hyperphosphorylated neurofibrillary tangles of tau protein, synaptic loss, and neuronal atrophy. Therefore, models have also been developed to study tauopathies, and have been used to study AD-related pathways alone or in combination with APP-overexpressing lines. Neuronal death has been demonstrated in some of these lines, theoretically making them optimal choices for the study of AD, although all models have caveats and drawbacks (Onos, et al., 2016). The pathological features present, as well as the timing and severity of progression, is highly dependent upon the mutations and copy number involved. Efforts to identify models of sporadic, late-onset AD are ongoing

in many research groups, although thus far none have been well-established, outside, perhaps, apoE4 targeted-replacement mice, which show cognitive decline in the absence of familial APP mutations (Rodriguez et al., 2013). A gargantuan effort spearheaded by the National Institute on Aging in conjunction with the University of Indiana, The University of California Irvine, Sage Bionetworks, and The Jackson Laboratory, seeks to establish more relevant models of AD (https://model-ad.org/).

Amyloid-centric models of AD include PDAPP (Games et al., 1995), Tg2576 (Hsiao et al., 1996), APP/PS1 (Perez-Tur et al., 1995; Jankowsky et al., 2001), J20 (Mucke et al., 2000), APP23 (Sturchler-Pierrat et al., 1997), 5xFAD (Oakley et al., 2006), and SwDI (Davis et al., 2004; Onos et al., 2016). The timing and severity of pathology in these animals varies widely. For instance, the 5xFAD model has an extremely early onset of pathology relative to the other models. This offers the advantage of performing studies on AD without the need to wait many months for mice to age. However, as AD is an age-related disease, the relevance to human disease is questionable, although strains with reduced APP expression levels have been developed to slow the onset of pathology in these animals (Oakley et al., 2006). In addition, the inclusion of 5 simultaneous AD-related familial mutations in APP is highly unlikely to be observed in any human patient. The APP/PS1 mouse model is one of the most widely used, and employs the overexpression of human APP harboring a familial AD mutation, as well as a mutation in presenilin (PS1), which is a component of the APP cleaving enzyme gamma secretase (Perez-Tur et al., 1995; Jankowsky et al., 2001). These animals produce elevated levels of the highly pathogenic variant of Aβ, $A\beta_{42}$, which is highly prone to aggregation, and show age-related amyloid deposition and cognitive impairment beginning around 6 months of age (Perez-Tur et al., 1995; Jankowsky et al., 2001). Interestingly, SwDI mice, harboring 3 mutations in APP associated with familial AD/dementia, offer a somewhat

unique CAA-centric model, with pronounced deposition of Aβ in the vasculature of the brain (Davis et al., 2004). It is important to note, however, that many other models, including Tg2576 and APP/PS1, do show varying degrees of CAA (Klohs et al., 2014).

In humans with AD, cognitive decline correlates much more closely with tau pathology than with that of Aβ (Brier et al., 2016). Therefore, the lack of overt tau pathology in the amyloidogenic models of AD is a significant drawback. Tau models have been developed to overcome this hurdle, and they include rTg4510, hTau, and PS19, utilize mutated forms of human tau associated with tauopathy (Terwel et al., 2005; Onos et al., 2016). These mice have been shown to develop intraneuronal hyperphosphorylated tau tangles, as well as neuronal atrophy (Andorfer et al., 2003; Andorfer et al., 2005; Ramsden et al., 2005). In order to more faithfully model the human AD brain, amyloid and tau models have been combined. The 3xTg model incorporates mutated forms of human APP, tau, and presenilin, and has been shown to develop both amyloid plaques and neurofibrillary tangles (Oddo et al., 2003). Importantly, cognitive decline in these animals correlates more strongly with tau pathology than with amyloid deposition, similar to observations made in human AD (Huber et al., 2017).

Models have also been developed to study AD risk genes and their influence on cognition and pathological progression, alone and in combination with APP-expressing lines. These models include knock in of human apoE isoforms, AD-related mutations in TREM2 and other GWAS hits, as well as knock out or overexpression of a wide range of implicated proteins including apoE, TREM2, ABCA1, apoA-I, and many more. Some of these lines will be discussed in greater detail later. Other models of sporadic AD include injection models, utilizing neurotoxic agents to degenerate cholinergic neurons, or even AD patient-derived Aβ to seed plaque formation (Gulyaeva et al., 2017).

Role of High-Density Lipoprotein in Alzheimer's Disease

Lipoproteins are the complexes of various lipids and proteins (Vance and Vance, 2008). Formed extracellularly, these soluble particles circulate in virtually all bodily fluids. The general structure of lipoprotein particles includes a hydrophobic core surrounded by a hydrophilic shell. The hydrophobic core contains neutral lipids, predominantly triglycerides (TGs) and cholesterol esters (CEs). The hydrophilic shell consists of primarily phospholipids (PL), unesterified free cholesterol (FC), and various apolipoproteins (apos), which mediate interactions with a variety of other molecules including enzymes, transporters, and receptors through a dynamic process. The main function of lipoproteins is facilitating the delivery and clearance of lipids and lipid-soluble or lipid-associating molecules throughout the body. Lipoproteins can be characterized by their size, density, electrophoretic mobility, and composition. The most commonly used classification of lipoproteins is by density. Due to the dynamic nature of lipoproteins, each class of lipoproteins can be divided into several subclasses. The focus of this review is on high-density lipoprotein (HDL), a heterogeneous group of lipoprotein particles with a density of 1.063–1.210 g/mL and size of approximately 7–20 nm. They are formed both in the systemic circulation and in the brain. Plasma HDL has been studied extensively because of its well established protective role in the cardiovascular system. Recent studies strongly suggest that the benefits of HDL extend to many other systems including the central nervous system (CNS). Mounting evidence indicates that HDL modulates cognitive function in aging and age-related neurodegenerative disorders, including AD.

High-density lipoprotein metabolism in the systemic circulation

Although HDL is often referred to as HDL cholesterol (HDL-C), apoA-I is the major protein component of HDL in the plasma and determines most of its functions (Segrest et al., 2000). In addition to apoA-I, plasma HDL also contains many other apos. Mature human apoA-I is a 243 residue polypeptide produced predominantly by the liver and intestine. The lipid-associating domain (residues 44–243) of human apoA-I contains tandem repeats of amphipathic α-helixes (Segrest et al., 1994). HDL biogenesis starts with the interaction between lipid-poor apoA-I and ATP-binding cassette transporter A1 (ABCA1) on the cell membrane of peripheral tissues, resulting in the formation of nascent discoidal HDL particles from cell membrane-derived PL and FC (Oram and Heinecke, 2005). Of note, other apos can also act as lipid acceptors for ABCA1. Importantly, this is the first step of reverse cholesterol transport (RCT), a process that removes excess cholesterol from peripheral tissues to the liver for excretion in the bile. Once these discoidal particles reach the plasma, apoA-I activates lecithin cholesterol acyltransferase (LCAT), forming mature, spherical, CE-rich HDL particles.

In the plasma, HDL interacts with cells and other lipoprotein particles through multiple receptors, transporters, and enzymes. Mature HDL can remove cholesterol from peripheral cells through other ABC transporters such as ABCG1 and ABCG4, further promoting RCT. Lipid- rich HDL selectively delivers CE to hepatocytes and steroidogenic cells through scavenge receptor B1 (SR-B1), regenerating lipid-poor apoA-I/HDL particles for further interaction with ABCA1. HDL-bound cholesteryl ester transfer protein (CETP) mediates the exchange of CE from HDL to non-HDL particles and the transfer of TG from TG-rich lipoproteins to HDL, resulting in a decrease of HDL-C levels. Thus, CETP inhibitors have been developed to raise HDL levels (discussed below). Other

major proteins that interact with HDL include phospholipid transfer protein (PLTP), endothelial lipase, and hepatic lipase (Vance and Vance, 2008).

It is well established that plasma levels of apoA-I/HDL are negatively correlated with the incidence of coronary heart disease in humans (Davidson and Toth, 2007). The mechanisms by which apoA-I/HDL protects against atherosclerosis are not fully understood at present. One of the major mechanisms is related to the role of apoA-I/HDL in RCT (Oram and Heinecke, 2005). The initial cholesterol efflux involving the interaction of apoA-I and ABCA1 is a critical step in RCT. Mutations/ polymorphisms on ABCA1 cause a significant reduction in HDL levels (familial hypoalphalipoproteinemia), to the point of near absence as reported in patients with Tangier disease (Oram and Heinecke, 2005).

In addition to its role in RCT, apoA-I/HDL exerts a wide range of other functions including anti-oxidation (Navab et al., 2000), anti-inflammation (Cockerill et al., 1995), pro-endothelial function (O'Connell and Genest, 2001), anti-thrombosis (Barter et al., 2004), and modulation of immune function (Barter et al., 2004). The multi-functionality of HDL contributes to its cardioprotective role. With the advance of modern technologies, recent proteomic and lipidomic analyses have revealed that approximately 188 proteins and over 200 lipid species are associated with plasma HDL (Toth et al., 2013). In addition, microRNAs (miRNAs) have also been found in human plasma HDL, and remarkably, HDL could deliver miRNAs to recipient cells through the SR-B1-dependent pathway (Vickers et al., 2011). Clearly, the complexity of HDL composition and function presents both the challenge and opportunity to develop HDL-based biomarkers and therapies for a number of diseases.

HDL metabolism in the central nervous system

While lipoprotein metabolism in the systemic circulation has been studied extensively, interest in lipoprotein metabolism in the brain has increased in recent years mainly because of connections between apoE and the development of several neurological disorders (discussed below). The brain contains ~25% of total body cholesterol, despite the fact that the it accounts for only 2% of total body mass (Dietschy and Turley, 2001). It is generally thought that there is no net transfer of cholesterol from the periphery into the CNS because the blood–brain barrier (BBB) restricts the movement of plasma lipoproteins into the brain. Thus, essentially all cholesterol in the brain comes from de novo synthesis. In adults, the rate of cholesterol synthesis exceeds the need for forming new structures. One of the excretory pathways involves the formation of 24S-hydroxycholesterol that crosses the BBB into the plasma (Dietschy and Turley, 2001).

The major apolipoprotein in the brain is apoE, primarily produced by glial cells. In humans, there are three isoforms of apoE coded by three alleles: APOE-ε2, APOE-ε3, and APOE-ε4, with an allele frequency of 7, 78, and 15%, respectively (Strittmatter and Roses, 1996). ApoE has received tremendous attention due to its genetic association with AD. While the APOE-ε2 allele confers some protection against AD (Corder et al., 1994), the APOE-ε4 allele is associated with an increased risk of AD (Corder et al., 1993; Poirier et al., 1993). The brain also expresses lipoprotein receptors (e.g., LDLR, LRP, and SR-B1), enzymes (e.g., LCAT and lipases), transfer proteins (e.g., PLTP and CETP), and ABC transporters (e.g., ABCA1 and ABCG1), although the presence of CETP in the brain is controversial (Albers et al., 1992; Demeester et al., 2000; Yamada et al., 1995). Because these proteins have well-established roles in cholesterol metabolism in the periphery, they are thought to play similar functions in the brain. HDL-like lipoprotein particles are found in the CSF and contain mainly apoE and apoA-I (Koch

et al., 2001; Pitas et al., 1987). While the source of apoE is clearly from glia as plasma apoE cannot cross the BBB (Linton et al., 1991), the origin of apoA-I in the CSF is uncertain. It is generally thought that the brain does not produce apoA-I and that apoA-I in the brain comes from the circulation (Dietschy and Turley, 2001). However, porcine cerebral endothelial cells have been shown to produce apoA-I (Mockel et al., 1994). Notably, the concentration of apoA-I in the CSF is comparable to that of apoE (Koch et al., 2001). In addition, plasma and CSF HDL cholesterol and apoA-I levels are correlated, suggesting that plasma apoA-I/ HDL levels can influence brain apoA-I/HDL levels (Fagan et al., 2000). While the role of apoE in brain cholesterol metabolism and other path- ways is well established (Yu et al., 2010), the neurobiological role of apoA-I has not been well studied. Experimental evidence has shown that rat astrocytes interact with both human apoE and apoA-I and generate HDL-like particles with distinct properties: apoE-HDL particles are cholesterol-rich whereas apoA-I-HDL particles are phospholipid-rich (Ito et al., 1999). Human CSF lipoproteins are capable of inducing a significant cholesterol efflux from rat astrocytes (Demeester et al., 2000).

The efflux ability of CSF lipoproteins is correlated more with the concentration of apoA-I in the CSF than that of apoE (Demeester et al., 2000). Also, exogenous human apoA-I is able to initiate a signal transduction pathway of intracellular cholesterol trafficking involving the activation of protein kinase C (PKC) in rat astrocytes for HDL biogenesis (Ito et al., 2002; Ito et al., 2004). In addition, apoA-I and apoE-containing HDL in the CSF go through different remodeling in response to traumatic brain injury in human (Kay et al., 2003). These findings, together with other evidence discussed below, suggest that apoA-I-containing HDL may have important functions in the brain under physiological and pathological conditions.

High-density lipoprotein and aging

While many genetic and environmental factors contribute to a healthy aging process, recent studies indicate that high density lipoprotein (HDL) may play a significant role in maintaining cognitive function during aging. A study with a group of 139 centenarians (Ashkenazi Jews older than 95 years) showed that plasma HDL levels were highly and positively correlated with cognitive function (Atzmon et al., 2002). Consistent with the HDL levels, increased plasma apoA-I and decreased plasma triglyceride levels were also correlated with a significantly superior cognitive function. Another study in 158 Ashkenazi Jews with exceptional longevity (average age 99 years) also found that high levels of HDL were associated with less age-related cognitive impairment and improved memory (Barzilai et al., 2006). In agreement, the Leiden 85-plus study with 561 subjects also reported that low HDL was associated with cognitive impairment independent of atherosclerotic disease (van Exel et al., 2002). A recent population-based study, the Longitudinal Aging Study Amsterdam, further showed that high HDL was associated with better memory performance in people aged 65 years and older (van den Kommer et al., 2012). Consistently, low HDL levels have been found to be associated with poor memory and decline in memory in middle-aged adults and cognitively normal elderly individuals in the Whitehall II study and the Sydney Memory and Aging study, respectively (Singh-Manoux et al., 2008; Song et al., 2012). These findings underscore the protective effects of increased plasma HDL and its role in maintaining superior cognition during aging.

Notably, known as a major genetic determinant for AD, the genotype of apoE also affects cognitive decline in normal aging. Carriers of the APOE-ε4 allele showed decline in memory before the age of 60 years and exhibited greater acceleration than non-carriers (Caselli et al., 2009). In aged individuals without dementia, possession of the APOE-ε4 allele predicts a higher rate of cognitive decline in the ninth decade of life

(Schiepers et al., 2012). It is also worth noting that carriers of APOE-ε4 have amore proatherogenic profile with lower HDL and higher VLDL and TG levels in the plasma than non-carriers. A recent study suggests that the high lipid affinity of apoE4 is responsible for such a lipid profile (Li et al., 2013). These findings suggest that besides the direct influence of apoE4 on brain function, systemic effects of apoE4 may also contribute to the compromised cognitive performance in carriers. Importantly, apoE4 carriers are also at an increased risk of death (Kulminski et al. 2014), an effect that was only observed in women between the ages of 70-95. Interestingly, the authors contend that only a small proportion of apoE4-associated deaths are explained by AD or cardiovascular disease (CVD), and that cancer plays a strong role in this observation. E4-carrier females with cancer had a 2-fold higher risk of mortality in this study.

In addition to APOE-ε4 allele, recent gene association studies provide further evidence for the beneficial effects of HDL and/or apoA-I on cognitive function in aging. Functional polymorphisms in the gene for cholesteryl ester transfer protein (CETP), which cause lower levels of CETP and higher levels of HDL, are associated with slower cognitive decline during aging (Barzilai et al., 2006; Izaks et al., 2012; Sanders et al., 2010), although some inconsistency exists (Yu et al., 2012). Furthermore, genetic variants in apoC-III, which cause lower levels of TG and higher levels of HDL, are associated with exceptional longevity (Atzmon et al., 2006) and cardioprotection (Jorgensen et al., 2014; Pollin et al., 2008; The et al., 2014), whereas the apoC-III variants that raise TG and lower HDL are associated with impaired cognition (Smith et al., 2009). Whole genome sequence-based analysis suggests that common variation contributes more to heritability of HDL levels than rare variation (Morrison et al., 2013). Whether all HDL-regulating genetic variations affect cognitive function awaits further investigation.

High-density lipoprotein and Alzheimer's disease

While aging itself is the biggest risk factor for AD, the APOE-ε4 allele is a major genetic risk factor for sporadic AD (Corder et al., 1993). The role of apoE in AD has been well studied, and reviewed in great detail (Holtzman et al., 2012; Kanekiyo et al., 2014; Mahley and Huang, 2012). In addition to APOE, recent large genome-wide association studies have identified over 20 loci that contribute to the risk of sporadic AD (reviewed in Reitz, 2012; Rosenthal and Kamboh, 2014). Several loci, such as CLU (clusterin or apoJ) and ABCA7, are closely involved in the cholesterol metabolism pathway. However, both CLU and ABCA7 also have roles in the innate immunity. Whether CLU and ABCA7 variants associated with the AD risk influence brain or plasma HDL levels or functions Is unknown. This section focuses on the relationship between plasma apoA-I-containing HDL and AD.

Clinical studies in different ethnic populations have shown that high levels of plasma HDL were associated with a decreased risk for AD, although there have been a few exceptions (Launer et al., 2001; Reitz et al., 2004; Vollbach et al., 2005). An early study with a group of 45 Japanese patients with AD found that plasma levels of apoA-I and apoA-II were markedly decreased compared to 79 controls (Kawano et al., 1995). Consistently, a study with a cohort of 98 French AD patients and 59 controls showed that decreased HDL cholesterol and serum apoA-I concentrations were highly correlated with the severity of AD (Merched et al., 2000). Another study with 334 elderly French subjects found that high HDL cholesterol levels were associated with a significantly decreased risk of AD (Bonarek et al., 2000). Furthermore, the Honolulu–Asia aging study with 929 men indicated that the levels of apoA-I and HDL cholesterol were inversely associated with the risk of AD (Saczynski et al., 2007). More recently, the Manhattan cognitive study with 1130 individuals also showed that high levels of HDL

cholesterol were associated with a decreased risk of both probable and possible AD (Reitz et al., 2010). Consistently, the InChianti study with 1051 Italians older than 65 years of age reported that low HDL cholesterol levels were associated with dementia (Zuliani et al., 2010). In addition, another recent study with 664 subjects from the Sydney Memory and Aging study reported that elderly individuals with mild cognitive impairment (MCI) had abnormal plasma levels of HDL-associated apos. MCI subjects had lower levels of apoA-I, apoA-II and apoH, and higher level of apoE and apoJ. Lower apoA-I, apoA-II and apoH levels increased the risk of cognitive decline over two years. Intriguingly, among the apos, apoA-I was the most significant predictor of cognitive decline (Song et al., 2012).

Further support for a protective role of HDL in AD comes from studies in animal models. Generally, mice are not an ideal animal model for studying human lipoprotein metabolism and AD, due to physiological differences between the species. However, many different lines of transgenic mouse models have been developed to better emulate relevant human physiology (Getz and Reardon, 2012; LaFerla and Green, 2012). Multiple laboratories have consistently shown that genetic and pharmacological manipulation of important players in HDL biogenesis pathways, such as ABCA1 and liver X receptors (LXRs), modifies the development of AD-like pathology and cognitive impairment in mouse models of AD (Burns et al., 2006; Donkin et al., 2010; Fitz et al., 2010; Hirsch-Reinshagen et al., 2005; Jiang et al., 2008; Koldamova et al., 2005a, 2005b; Riddell et al., 2007; Vanmierlo et al., 2011; Wahrle et al., 2005; Wesson et al., 2011; Zelcer et al., 2007). Furthermore, genetic overexpression of human apoA-I and accompanied increase of functional HDL prevented the development of age-related cognitive deficits in the APP/PS1 mouse model of AD (Lewis et al., 2010). Consistently, lack of apoA-I exacerbated cognitive deficits in APP/PS1 mice (Lefterov et al., 2010).

Intriguingly, genetic manipulation of apoA-I does not affect total brain parenchymal Aβ deposition (Fagan et al., 2004; Lefterov et al., 2010; Lewis et al., 2010) but significantly changes the dynamics of cerebrovascular Aβ deposition; apoA-I overexpression attenuates whereas apoA-I deficiency exacerbates cerebral amyloid angiopathy (CAA) in AD mice (Lefterov et al., 2010; Lewis et al., 2010). Notably, HDL deficiency could be particularly detrimental in APOE-ε4 carriers as a recent study showed that ABCA1 deficiency worsened AD-like cognitive impairment and Aβ deposition in human apoE4 but not in apoE3-targeted replacement mice (Fitz et al., 2012). In apoE4 mice, plasma HDL and Aβ levels were significantly decreased and the plasma HDL level was negatively correlated with amyloid plaques in the brain, suggesting a role of plasma HDL in Aβ clearance. Taken together, these findings provide compelling evidence that HDL and associated apolipoproteins play a pivotal role in modulating the pathogenesis of AD.

Mechanisms by which HDL may modulate cognitive function

Although the evidence for the protective role of HDL in cognition is substantial, the underlying mechanisms by which HDL modulates cognitive function are poorly understood. Clearly, multiple functions of HDL are involved under different conditions. To simplify the discussion, AD is used to illustrate potential mechanisms of action for apoA-I to modulate the disease process. Since the systemic effects of HDL are well established (Davidson and Toth, 2007) and the cerebrovascular function of HDL in AD has been summarized recently by an excellent review (Stukas et al., 2014), this section focuses on the potential direct role of apoA-I in the brain.

Cholesterol efflux pathway

It has been shown *in vitro* and *in vivo* that, as in the periphery, apoA-I in the brain promotes the cellular cholesterol efflux through ABCA1 and forms discoidal HDL-like

particles (Ito et al., 1999; Wahrle et al., 2004). With the activation of LCAT by apoA-I, FC is converted to CE, resulting in the formation of spheroidal HDL-like particles. These particles are cleared by interacting with receptors such as SR-B1 by cells in the brain or through the BBB to peripheral circulation (Panzenboeck et al., 2002). They also function to deliver cholesterol to sites for growth or healing (Kay et al., 2003). While it is true that most apolipoproteins can act as cholesterol acceptors in ABCA1-mediated cholesterol efflux, they exhibit differential efficacy and produce particles with distinct properties (Ito et al., 1999). It has been shown that apoA-I in the CSF is more efficient than apoE for mediating cholesterol efflux (Demeester et al., 2000).

APP trafficking and processing pathway

APP trafficking and processing are modulated by a number of mechanisms (Cam and Bu, 2006; Haass et al., 2012; Small and Gandy, 2006). One of the mechanisms is cell membrane fluidity, regulated mainly by the cholesterol content. While the non-amyloidogenic cleavage of APP by α-secretase occurs in cholesterol-poor and phospholipid-rich domains, the amyloidogenic cleavages by β-and γ-secretases are preferred in the cholesterol-rich domains (lipid rafts) (Wolozin, 2001). Another controlling mechanism for APP processing is the distinct localization of secretases. Theα-secretase activity is located primarily at the cell surface, whereas β-and γ-secretase activities are found mainly in membranous compartments (e.g., endosomes) inside the cell (Cam and Bu, 2006; Haass et al., 2012; Small and Gandy, 2006). Therefore, apoA-I/HDL in the brain may affect the APP processing pathways through both of the following mechanisms: a) apoA-I mediates efficient cellular cholesterol efflux (Demeester et al., 2000); the resultant increase in membrane fluidity could enhance α-secretase cleavage of APP at the cell membrane and b) apoA-I binds to APP at the cell surface (Koldamova et al., 2001); thereby it may prevent APP from undergoing the endocytic process, which

is necessary for β-and γ-secretases to access APP. Thus, the final consequence of these effects would be reduced generation of Aβ.

Aβ clearance pathway

Overproduction of Aβ in the brain causes familial AD, but impaired Aβ clearance from the brain is implicated in sporadic AD (Castellano et al., 2011; Mawuenyega et al., 2010; Scheuner et al., 1996). ApoA-I binds to Aβ and inhibits Aβ aggregation and cytotoxicity *in vitro* (Koldamova et al., 2001). In addition, the binding affinity of human apoA-I for Aβ is higher than that of human apoE (Koldamova et al., 2001). Therefore, the apoA-I/HDL in the brain is expected to be more effective in binding Aβ and mediates the clearance of Aβ by local cells (e.g., astrocytes and microglia) through the scavenger receptor (e.g., SR-B1) and/or by crossing the BBB to the systemic circulation (Sagare et al., 2012). Supporting this notion, studies in AD mice have demonstrated that lack of apoA-I exacerbates whereas overexpression of human A-I ameliorates cerebrovascular deposition of Aβ (Lefterov et al., 2010; Lewis et al., 2010). Recently, it was shown that cholesterol-containing lipid membranes promote the nucleation of Aβ aggregates (Habchi et al., 2018), and in iPSCs derived from humans expressing either apoE3 or apoE4, it was demonstrated that apoE4 cells express greater levels of transcripts associated with cholesterol biosynthesis, while they downregulate genes responsible for cholesterol clearance (https://www.alzforum.org/news/research-news/apoe-has-hand-alzheimers-beyond-av-beyond-brain). It has also been shown that apoE has minimal direct interaction with Aβ and competes with Aβ for the same clearance pathways within the brain (Verghese et al., 2013). These intriguing results suggest that upregulation of apoA-I and/or inhibition of apoE competition with Aβ for cellular uptake in the brain might be an effective means to enhance Aβ clearance.

Anti-oxidation and anti-inflammation

Oxidative stress and inflammation contribute to the etiology of AD (Keeney et al., 2013; Schrag et al., 2013; Wyss-Coray and Rogers, 2012). Anti-oxidant and anti-inflammatory properties of apoA-I/HDL have been shown to play significant roles in protecting against cardiovascular disease (Barter et al., 2004). These same mechanisms may play a significant role in neuroprotection. Previous studies support this hypothesis: a) the level of CSF apoA-I is increased significantly after infection in macaques (Saito et al.,1997); b) CSF apoA-I-containing lipoproteins remodel after traumatic brain injury in humans (Kay et al., 2003); c) reconstituted human apoA-I-containing HDL reduces neuronal damage in rat models of stroke, via an anti-oxidative mechanism (Paterno et al., 2004); d) an apoA-I mimetic peptide inhibits inflammation in the brain and improves cognitive performance in mice (Buga et al., 2006; Handattu et al., 2009); and e) overexpression of human apoA-I attenuates neuroinflammation in AD mice (Lewis et al., 2010). The importance of inflammation to AD pathology as well as the potential of HDL to mitigate that pathology will be discussed in greater detail later in this literature review.

Signal transduction and synaptic plasticity

Aβ-induced synaptic dysfunction is thought to be the underlying cause for cognitive impairment in AD (Selkoe, 2002). ApoA-I/HDL has been shown to activate several kinases (e.g. PKA, PKC, PI3K, MAPK, and Akt) and increase the level of cAMP directly or indirectly through ABCA1 or SR-B1 in peripheral cells and in astrocytes (Haidar et al., 2004; Ito et al., 2004; Mineo et al., 2003; Yamauchi et al., 2003). These molecules play important roles in signaling pathways pertinent to synaptic function and memory formation. ApoA-I may directly modulate synaptic plasticity through interactions with these signaling molecules. ApoA-I and HDL may influence signaling in other cell types

as well; of particular interest is the recently discovered interaction between apoA-I and TREM2, an AD risk-associated transmembrane receptor that signals through several kinase pathways to control microglial function and survival (Bailey et al., 2015; Atagi et al., 2015; Yeh et al., 2016). This interaction and its relevance to AD is discussed in greater detail below.

High-density lipoprotein–enhancing pharmacotherapies

As discussed in previous sections, compelling evidence indicates that functional HDL is crucial for the protection of cardiovascular, cerebrovascular, and cognitive functions. Thus, therapeutic approaches that enhance HDL functions will benefit both peripheral and central nervous systems. Although exercise, diet and other lifestyle measures are the most favorable ways to raise HDL levels, adherence to these measures might be difficult to achieve. Furthermore, there are genetic conditions in which lifestyle change alone may not be sufficient to modulate the level and function of HDL. In these scenarios, therapeutic intervention is needed. This section summarizes HDL-enhancing pharmacotherapies currently available or under investigation.

Exercise

Endurance exercise has been shown to improve HDL levels (Couillard et al., 2001), and is commonly recommended as a lifestyle intervention in cardiovascular diseases such as diabetes (Yang et al., 2014) and atherosclerosis (Palmefors et al., 2014). The impact of physical exercise on cognitive function and AD risk are of particular interest within the scope of this dissertation. A recent clinical study found that Swedish women who exercised regularly in mid-life were 90% less likely to develop dementia later in life, when compared with those who achieved a lower cardiovascular fitness (Hörder et al., 2018). However, a recent trial found no benefit for participants with mild-to-moderate

dementia who engaged in cardiovascular exercise twice-weekly for 1 year (Lamb et al., 2018). Whether exercising more than twice per week might provide benefit in these patients, as it did in reducing risk in the Swedish cohort, is unclear.

Niacin and niacin receptor agonists

Niacin, also known as vitamin B3 or nicotinic acid, is an important precursor for the coenzymes NAD and NADP, which are essential for proper tissue catabolism and anabolism. GPR109A (PUMA-G/HM74A) was identified as the receptor for niacin (Tunaru et al., 2003). GRP109A is a G-protein coupled receptor expressed in adipocytes, spleen, and immune cells. When activated, GRP109A reduces intracellular cAMP and inhibits lipolysis.

Niacin has been used for over 60 years to raise HDL-C levels (Carlson, 2005). At present, niacin is the most effective HDL-raising agent available clinically. It also lowers the level of TG, lipoprotein(a), and LDL-C (Toth et al., 2013). A recent clinical trial (AIM-HIGH) showed that in patients with cardiovascular disease and low HDL-C levels, treatment with extended release niacin, 1500 to 2000 mg per day, significantly increased HDL-C (25%) while decreasing TG (29%) and LDL-C (16%) (Investigators et al., 2011). Further analysis also showed that niacin treatment modestly increased apoA-1 (7%), decreased apoB (13%), decreased the apoB/apoA-I ratio (19%), and decreased lipoprotein(a) (21%) (Albers et al., 2013). However, these favorable changes in lipoprotein profiles did not lead to the reduction of cardiovascular events. It is worth noting that the patients in this trial were receiving intensive statin therapy and their baseline LDL-C was very low (74 mg/dL) (Investigators, 2011). Thus, it is possible that no additional benefits from niacin treatment can be achieved in patients with very low LDL-C levels. This possibility is supported by another recent clinical trial (HPS2-

THRIVE) (Group, H.T.C., 2013). In this study, participants were treated with extended release niacin combined with laropiprant, a prostaglandin-D2 receptor-1 inhibitor, to alleviate niacin-induced facial flushing. Subgroup analysis from this study showed that in participants with LDL-C lower than 78 mg/dL no benefit was found with niacin/ laropiprant treatment, but in participants with LDL-C higher than 78 mg/dL benefit was observed with the treatment (Group, H.T.C., 2013). It is worth noting that the formulation of niacin influences the side effects observed. Standard immediate release niacin causes a high frequency of flushing and long-acting niacin causes less flushing but increases the risk of hepatotoxicity, whereas extended release niacin causes fewer of both types of adverse effects (McKenney, 2003). Thus, a proper formulation of niacin should be selected to reduce potential side effects.

In addition to niacin, synthetic GRP109A agonists, such asMK-1903, have been developed. MK-1903 has been evaluated in phase I and II studies to treat dyslipidemia. MK-1903 treatment produced a significant decrease in plasma free fatty acids. However, MK-1903 had a smaller effect on serum lipid levels compared with niacin, suggesting that niacin may act on a GRP109A-independent pathway (Boatman et al., 2012). Further studies in animal models and humans confirmed that GPR109A receptor does not mediate niacin's lipid efficacy (Lauring et al., 2012), opening the door for identifying new molecular target(s) of niacin and developing novel approaches to raise HDL.

It has been suggested that Niacin intake is inversely correlated with AD risk (Morris et al., 2004), although the therapeutic potential of increased niacin intake has not been studied in AD patients.

PPARα agonists — fibrates

There are four commonly prescribed drugs in the fibrate family: bezafibrate, ciprofibrate, gemfibrozil, and fenofibrate. Fibrates mainly work by activating the peroxisome proliferator-activated receptor α (PPARα). Activation of PPARα induces the transcription of genes that promote lipoprotein lipolysis, decrease TG production, facilitate LDL clearance, reduce CE and TG exchanges between VLDL and HDL, and increase HDL/apoA-I production (Staels et al., 1998). Thus, fibrates are used in patients with low HDL-C or high TG levels. However, mixed results have been reported from clinical trials with fibrates for cardiovascular diseases (reviewed in Toth et al., 2013). Post-hoc analyses of multiple trials suggest that fibrates produce significant benefits only in subgroups of patients with low HDL-C and high TG levels. Interestingly, in a group of 22 elderly hypertriglyceridemic patients, 600 mg of gemfibrozil daily resulted in a significant decrease in serum TG levels. Patients treated with gemfibrozil maintained better cerebral perfusion and scored better on cognitive performance measures than untreated controls (Rogers et al., 1989). Cognitive benefits of fibrates need to be confirmed in further clinical studies.

In addition, fibrates are commonly used in combination therapy with stains. It is important to note that the combination of gemfibrozil and statin significantly increases the risk of rhabdomyolysis (Pierce et al., 1990; Staffa et al., 2002), due to partial inhibition of gemfibrozil on the metabolism of statins (Prueksaritanont et al., 2002). In contrast to gemfibrozil, fenofibrate does not increase the concentrations of statins (Bergman et al., 2004). The combination of fenofibrate and statin has been used in large, long-term clinical trials and there was no evidence for an increased risk of myositis or rhabdomyolysis compared to statin monotherapy (Farnier et al., 2011; Group, A.S. et al., 2010). A recent meta-analysis on the safety of the coadministration of statin with

fenofibrate also concluded that statin-fenofibrate combination therapy was tolerated as well as statin monotherapy (Guoet al., 2012).

The role of fibrates in modifying AD pathology has not been well explored. In the N2a neuroblastoma cell line, it was shown that fenofibrate reduces the extracellular proteolytic degradation of Aβ, the opposite of which was observed with fenofibric acid (Abdul-Hay et al., 2009). However, a more recent *in vivo* study, using APP/PS1 mice, demonstrated that fenofibrate reduces BACE1 mRNA and protein levels, as well as Aβ levels (Zhang et al., 2014). The differences observed *in vitro* and *in vivo* may be due, in part, to the influence of glial and endothelial cells on amyloid clearance from the brain.

CETP inhibitors

Based on several lines of evidence that CETP deficiency/inhibition is associated with an elevated level of HDL and a decreased risk for cardiovascular disease, CETP inhibitors have been developed and tested in clinical trials. Torcetrapib was the first CETP inhibitor tested. In ILLUMINATE trial (Barter et al., 2007), torcetrapib significantly increased the level of HDL-C in treated patients but failed to show a clinical benefit. In fact, torcetrapib was associated with an increase in cardiovascular events due to unexpected off-target adverse effects resulting in hypertension. Dalcetrapib was the second CETP inhibitor to undergo clinical trials. In the dal-OUTCOMES trial (Schwartz et al., 2012), dalcetrapib successfully increased HDL-C levels but did not reduce recurrent cardiovascular events. Dalcetrapib was safe and the reason for its failure is not clear. It has been suggested that dalcetrapib-induced increase in HDL-C levels might not have been sufficient or it was not accompanied by an enhancement of the protective properties of HDL (Rader and de Goma, 2014; Toth et al., 2013). Two new CETP inhibitors, anacetrapib and evacetrapib, are much more potent than dalcetrapib and do not have the off-target

adverse effects of torcetrapib (Gotto et al., 2014; Nicholls et al., 2011). A recent large clinical trial of anacetrapib showed statistically significant reduction of adverse cardiovascular outcomes, and a concomitant increase of HDL-C, when compared to the placebo group (HPS3/TIMI55–REVEAL Collaborative Group et al., 2017). Nevertheless, Merck opted not to file for approval from the FDA for this agent, owing to a moderate effect size, failure to impact secondary outcomes, and safety concerns (http://investors.merck.com/news/press-release-details/2017/Merck-Provides-Update-on-Anacetrapib-Development-Program/default.aspx).

Reverse cholesterol transport (RCT) enhancers — liver X receptor agonists and retinoid X receptor agonists

As RCT is thought to be the most relevant cardioprotective mechanism mediated by HDL, much effort has been made to develop agents that promote RCT. Liver X receptors (LXRα and LXRß) are oxysterol activated nuclear receptors. Together with retinoid X receptors (RXRs), LXRs regulate the expression of a variety of target genes that control lipid and glucose homeostasis, steroidogenesis and inflammatory responses. Activation of LXRs has been shown to promote RCT through ABCA1 and ABCG1 and increase intestinal HDL generation (Brunham et al., 2006; Costet et al., 2000). Several synthetic LXR agonists, including T0901317, GW3965 and

Recently, accumulating pre-clinical evidence indicates the therapeutic potential of LXR agonists for AD. Studies in multiple laboratories have shown that LXR agonists improve cognitive functions either with or without reducing Aβ levels in the brain of AD mice (Donkin et al., 2010; Fitz et al., 2010; Jiang et al., 2008; Riddell et al., 2007; Vanmierlo et al., 2011; Wesson et al., 2011). Specifically, in the APP23 mouse model of AD, T0901317 treatment ameliorated amyloid pathology and memory deficits (Fitz et al.,

2010). It was shown that T0901317 treatment resulted in a decrease in Aβ levels in the interstitial fluid of the hippocampus, supporting the role of LXR agonists in facilitating Aβ clearance.

In vitro experiments demonstrated that ABCA1was essential for lipidation of apoE and mediated the effects of T0901317 on Aβ degradation by microglia (Fitz et al., 2010). The specific role of ABCA1 in mediating benefits of LXR agonists in AD mice was further confirmed by another study with GW3965 in the APP/PS1 mouse model of AD (Donkin et al., 2010). Recently, T0901317 was shown to mitigate apoE4-induced cognitive impairments, reduced amyloid oligomers (but not total amyloid burden), and increased apoE lipidation in APP/PS1 mice haplodeficient for ABCA1 and expressing either apoE3 or E4 (Carter et al., 2017). These findings indicate that LXR agonists exert neurological benefits through the ABCA1/apoE-HDL pathway. Interestingly, it has been demonstrated that GW3965 treatment dramatically increased the level of apoA-I in the brain of APP/PS1 mice independent of ABCA1 (Stukas et al., 2012). Therefore, increase of apoA-I/HDL may also contribute to the beneficial effects of LXR agonists in AD mice.

In addition to LXR agonists, emerging evidence indicates that RXR agonists may also possess the therapeutic potential for AD. In a highly publicized report, acute treatment with a RXR agonist, bexarotene, a drug currently approved for the treatment of cutaneous T-cell lymphoma, rapidly and dramatically decreased Aβ levels/plaques in the brain of AD mice (Cramer et al., 2012). Bexarotene treatment lowered soluble Aβ levels in mouse interstitial fluid by 25% within 24 hours and reduced Aβ plague area by more than 50% within 72 hours. It was shown that bexarotene increased Aβ clearance via an apoE-dependent mechanism as the treatment promoted the expression of apoE, ABCA1, and ABCG1 in the brain. Remarkably, bexarotene rescued cognitive function in a mouse model of AD after as few as 7 days of treatment (Cramer et al., 2012).

However, the effectiveness of bexarotene in AD mice has been questioned by subsequent studies as the reduction of Aβ plaques in treated mice could not be reproduced (Fitz et al., 2013; Price et al., 2013; Tesseur et al., 2013; Veeraraghavalu et al., 2013). The discrepancy observed in these studies might result from differences in drug formulations and mouse models (Landreth et al., 2013). Nevertheless, some studies replicated the decrease in soluble Aβ levels (Fitz et al., 2013; Veeraraghavalu et al., 2013) and the improvement of cognitive function (Fitz et al., 2013; Tesseur et al., 2013) in bexarotene-treated AD mice. Importantly, bexarotene increased Aβ clearance and rescued cognitive function in APP/PS1 mice expressing either human apoE3 or apoE4 isoform (Fitz et al., 2013). In contrast, amore recent study did not find any changes in Aβ plaques or cognitive deficits in bexarotene-treated APP/PS1 mice (LaClair et al., 2013). These inconsistencies were further evidenced by mixed results in the clinic, where bexarotene demonstrated reduction in amyloid levels in a small phase 2 placebo-controlled trial, but only in those not carrying apoE4 (Cummings et al., 2016). The authors highly recommended against using bexarotene off-label, due to known cardiovascular risks associated with elevated triglyceride levels. Thus, further studies are required to clarify the effects of bexarotene on AD-related processes, and whether triglyceride-lowering agents used in concert with bexarotene might enhance the safety and efficacy of this agent.

ApoA-I upregulators

RVX-208 is a novel small molecule that stimulates apoA-I gene expression leading to an increase in HDL levels and functionality (Bailey et al., 2010). It has been demonstrated that RVX-208 is a specific inhibitor for BET bromodomains that regulate expression of a variety of genes including apoA-I (McLure et al., 2013; Picaud et al., 2013). Early testing in African green monkeys demonstrated that a 63-day RVX-208 treatment markedly

increased serum apoA-I (60%) and HDL-C levels (97%), accompanied by the enhancement of ABCA1-, ABCG1-, and SR-B1-mediated cholesterol efflux (Bailey et al., 2010). Positive results from animal models and early human clinical trials have led to further human clinical trials. The Phase 2b SUSTAIN trial (NCT01423188) was designed to evaluate the lipid efficacy, safety and tolerability of RVX-208, and the ASSURE trial (NCT01067820) was designed to evaluate the effect of RVX-208 on atherosclerotic plaque burden using intravascular ultrasound (IVUS) imaging (Nicholls et al., 2012). Findings from these clinical trials suggest that RVX-208 has the potential for the treatment of cardiovascular disease. In addition, RVX-208 may also have the therapeutic potential for diabetes and AD. A Phase 3 clinical trial of RVX-208 in diabetic patients with low HDL levels is ongoing (https://clinicaltrials.gov/ct2/show/NCT02586155). In AD, a pilot Phase 1a study showed a trend towards increase in the level of Aβ40 in the plasma of patients treated with RVX-208 for 7 days compared to controls (http://www.resverlogix.com/upload/latest_news/81/01/2008-11-10_alzheimers_program_final.pdf). This preliminary result was confirmed in amore re-cent Phase II ASSERT trial, in which 12-week treatment with RVX-208 significantly increased the plasma Aβ40 level compared to baseline or the level of placebo-treated controls (http://www.resverlogix.com/media/press-release.html?id=451). These intriguing observations are consistent with the findings from genetic upregulation of apoA-I in AD mice (Lewis et al., 2010) and support the notion that elevating apoA-I levels in the systemic circulation enhances Aβ clearance from the brain. Further clinical trials of RXV-208 in AD or pre-AD patients will be needed to determine whether RVX-208 can modulate the progression of AD.

ApoA-I infusion

The strong negative correlation between plasma apoA-I levels and cardiovascular disease and consistent experimental results in animal models have led to direct infusion of apoA-I in human clinical trials. Nissen et al. infused human patients with a recombinant apoA-I Milano, a form of apoA-I that is associated with lower risk for cardiovascular disease (Franceschini et al., 1980), and observed a significant regression of coronary atherosclerosis (Nissen et al., 2003). Another group performed a randomized human trial to test the infusion of apoA-I incorporated into recombinant HDL (rHDL). The study determined that short-term infusion of rHDL produced a significant reduction of atheroma volume and improved plaque characterization index and coronary score (Tardif et al., 2007). More evidence of atheroprotection was obtained with the infusion of apoA-I or apoA-I Milano in both animal models and human clinical trials. Remarkably, a single infusion was shown to be enough to significantly reduce atherosclerosis and infer positive effects on plaque characterization (Tardif, 2010). Additionally, researchers infused rabbits with either lipid free apoA-I or apoA-I in rHDL. The infusions markedly inhibited vascular inflammation in the rabbits (Patel et al., 2010). A recent study found that infusion of apoA-I produced an increase in cholesterol efflux from macrophages, favorably remodeled HDL and reduced cytokine secretion in both rabbit and human blood (Diditchenko et al., 2013).

Whether apoA-I infusion has any effect on human cognition has not been investigated. As discussed in previous sections, low levels of apoA-I have been associated with poor cognitive function in aging and in neurodegenerative diseases. Experimentally, apoA-I overexpression in the periphery was shown to reduce neuroinflammation, attenuate cerebral amyloid angiopathy and inhibit cognitive decline in a mouse model of AD (Lewis et al., 2010). Our finding was recently supported by a study using the infusion of human

apoA-I Milano in the APP23 mouse model of AD, which demonstrated that i.v. infusion of apoA-I Milano reduces cerebral amyloid deposition and glial activation (Fernández et al., 2017). Thus, a beneficial effect of apoA-I infusion on cognitive function is an intriguing possibility, which merits further research.

HDL-mimetic peptides as potential therapeutics for Alzheimer's disease

The use of small peptides designed to mimic key proteins involved in HDL function, designated HDL-mimetic peptides, has been an area of great interest in the cardiovascular field since the 1980's (Anantharamaiah et al., 1986). This idea stems from the fact that lack of oral bioavailability, post-translational modification, and complex tertiary structure that is difficult and costly to recapitulate with synthesized proteins are major obstacles in the path of developing native or recombinant HDL, apos, or other HDL-associated proteins for use as therapeutic agents. Thus, orally bioavailable small peptides, which retain the atheroprotective effects of their parent proteins, have been developed. These peptides are modeled after a specific apolipoprotein and typically retain the consensus sequences and/or structural similarities of key motifs, such as a lipid- or receptor-binding region (Corijn et al., 1993; Labeur et al., 1997; Mishra et al., 1998). Mimetics can be synthesized from D-amino acids and thus have higher oral bioavailability, and methods to improve oral delivery of these mimetics have been described (Navab et al., 2009). Due to their relative physiological contributions to HDL function, and the degree to which they have been studied, the most robust efforts have been focused on the creation of mimetic peptides derived from apoA-I, apoE, and apoJ. Other apos have been used as a model for mimetics (Petraki et al. 2009; Amar et al., 2015; Kong et al., 2017), but they have not been well studied.

ApoA-I Mimetics

The importance of apoA-I in atherosclerosis and CVD led to a series of early efforts in developing apoA-I mimetics. The general design of the apoA-I mimetics is an amphipathic peptide, which adopts an alpha helical secondary structure similar to that seen in the full-length apoA-I (Segrest et al., 1992). A number of mimetic peptides were created and tested for therapeutic benefit in mice and cell culture. In order to preserve lipid-binding and anti-atherosclerotic activity, an 18 amino acid peptide was designed with structural, but not sequence, homology to apoA-I. The peptide, called 18A, forms an amphipathic alpha helical secondary structure and was shown to have a similar lipid-binding capacity as full-length apoA-I (Anantharamaiah et al., 1985).

A series of modifications were made to 18A, wherein a number of non-polar residues were replaced with phenylalanines (F) in an attempt to bolster its atheroprotective affects, and the most successful of these modified peptides was called 4F (Ac-DW**F**KA**F**YDKVAEK**F**KEA**F**-NH_2) (Segrest et al., 1983; Datta et al., 2001). The oral bioavailability of 4F in the plasma was quite low; and thus its enantiomer, D-4F, was created and shown to remain in the plasma for much longer after oral gavage (Navab et al., 2005a). A recent report indicates that the addition of polyethylene glycol (PEG) to apoA-I mimetic peptides increases their half-lives without impacting the ability of these peptides to mediate cholesterol efflux (Li et al., 2018).

The cardioprotective, anti-atherogenic, and anti-inflammatory efficacy of 4F has been well-described *in vitro*, in animal models, and in human clinical trials. D-4F has been shown to inhibit atherosclerotic lesion development and also to reduce inflammation in mice and rabbits (Navab et al., 2005a; Van Lenten et al., 2007; Morgantini et al., 2010;

Ou et al., 2012). The mimetic peptide remodels HDL into lipid-poor HDL, promotes RCT (Navab et al., 2005a), inhibits LDL aggregation (Nguyen et al., 2015), induces functional changes in macrophage activity (Symthies et al., 2010), improves arterial healing (Rosenbaum et al., 2015), reduces cardiac hypertrophy (Han et al., 2016), ameliorates pulmonary hypertension (Sharma et al., 2014), and reduces oxidative damage (Baotic et al., 2013; Liu et al., 2014), protecting against endothelial and macrophage apoptosis (Liu et al., 2017; Tian et al., 2017); Modification of 4F to more closely resemble the natural structural repeats observed in full-length apoA-I showed mixed results. Tandem repeats of 4F were found to possess superior efficacy in remodeling HDL and promoting cholesterol efflux, however, monomeric 4F more robustly cleared turbid lipid solutions, and showed stronger anti-oxidative capacity (Wool et al., 2008).

Phase 1 clinical trials showed that multiple doses of D-4F, administered orally, were well tolerated and improved the HDL anti-inflammatory profile of human patients with cardiovascular disease (Bloedon et al., 2008; Dunbar et al., 2017). These data make further studies on D-4F a particularly intriguing objective. A third phase 1 trial was conducted in which the L-4F peptide, injected either intravenously or subcutaneously, was found to be safe and well-tolerated, but did not show efficacy in improving the anti-inflammatory capacity of HDL *in vivo*, although *ex vivo* studies supported an anti-inflammatory effect of 4F (Watson et al., 2011). Interestingly, these data were addressed by Dunbar and colleagues in their more recent study, wherein they found that the oral dosage of 4F correlated more strongly with the extent of HDL anti-inflammatory capacity than did the plasma concentration achieved (Dunbar et al., 2017). The authors argued that enteric exposure is therefore more important in driving 4F-mediated beneficial effects than is plasma concentration.

In support of this conclusion, the effect of 4F in promoting RCT has been shown to be mediated by trans-intestinal cholesterol efflux, which is thought to account for nearly one-third of homeostatic clearance of sterols from the circulation (Meriwether et al., 2016). Further, another derivative of 18A, 6F, was shown to reduce atherosclerosis in an animal model after oral delivery, despite being detectable only in the intestine, and not the plasma (Chattopadhyay et al., 2013). Similarly, 4F was shown to reduce atherosclerosis to the same extent when administered either orally or subcutaneously (s.q.), despite approximately 1,000-fold greater plasma concentration in the animals receiving the peptide s.q. (Navab et al., 2011). In these animals, the intestinal concentration of 4F, as measured in the feces, was similar between the two routes of administration.

Due to the well-established correlation between vascular risk factors and cognitive decline, HDL mimetic peptides have been tested for efficacy in improving mental health. In fact, D-4F has been shown to have effects on cognitive capacity. In LDL receptor-null mice, oral D-4F was shown to reduce inflammation in the vasculature of the brain and improve cognitive performance without influencing plasma lipid levels (Buga et al., 2006). Preconditioning of rats with daily s.q. dosing of the 4F peptide was found to protect against neuronal damage in a model of brain injury (Yan et al., 2015). In apoE-null mice, intravitreal injection of 4F was recently found to mitigate age-related deposition of esterified cholesterol in the eye (Rudolf et al., 2018). Further, i.p. injection of D-4F was shown to reduce both acute and chronic inflammation, and associated pain, in rats with experimentally-induced arthritis (Oehler et al., 2017). Most relevant to this dissertation, oral D-4F, in combination with pravastatin, was shown to inhibit Aβ plaque formation and improve cognitive function in a mouse model of AD by inducing an anti-inflammatory effect in the brain (Handattu et al., 2009). D-4F did not impact plasma

HDL-C levels in this study, suggesting that the peptide improves HDL quality, not quantity, and/or directly modulates disease-related processes in the brain. Further studies on 4F and other HDL mimetic peptides are needed to illuminate their potential use as therapeutics in neurological disorders. Whether enteric exposure to 4F is important in mediating the AD-related effects of 4F has not been established.

Other apoA-I mimetic peptides have been developed, and also show vascular-protective effects (Tabet et al., 2010; Di Bartolo et al., 2011; Bucci et al., 2012; Ditiatkovski et al., 2013; Yahiro et al., 2014; Zhao et al., 2014; Amar et al., 2015; Shimizu et al., 2015; Suematsu et al., 2016; Takata et al., 2016), although their potential in neurological disease has not been tested. The use of a radiolabeled apoA-I mimetic as a positron emission tomography (PET) ligand, to non-invasively detect atherosclerotic lesions *in vivo*, has also been described (Kawachi et al., 2013).

ApoE Mimetics

In addition to apoA-I mimetics, recombinant HDL and peptides derived from other HDL-associated apos, including apoE and apoJ have also been created. As the most important and well-studied apo in the brain, apoE is an enticing target and the development of peptides to mimic its key functions has been undertaken by several groups. The need for apoE mimetics is furthered by the fact that full-length apoE does not cross the BBB (Linton et al. 1991), hindering the potential for apoE infusion as a therapeutic in neurological disorders, which has been attempted for apoA-I. Promisingly, mimetic peptides derived from the receptor-binding region of apoE have been shown to cross the BBB (Garber et al. 2003, Gupta et al. 2005; and Lynch et al. 2005; Lei et al., 2016).

CS-6253, a mimetic peptide derived from the c-terminus of apoE, has been shown to enhance ABCA1-mediated lipid efflux, promote RCT (Hafiane et al., 2015), and influence endogenous apo levels in the plasma and in the brain (Boehm-Cagan et al., 2016a). It has further been shown that CS-6253 improved apoE lipidation, reduced amyloid and tau pathology, and mitigated apoE4-driven cognitive impairment in mice (Boehm-Cagan et al., 2016b).

The smallest of the HDL mimetics peptides yet described, CN-105 is 5 amino acids in length, derived from the polar face of the receptor binding region of apoE (Lei et al., 2016). CN-105 has improved BBB permeability, when compared to other apoE mimetics, and has been shown to reduce neuroinflammation, and to improve survival and functional outcomes in mouse models of ischemic stroke, TBI, and intracranial hemorrhage (Lei et al., 2016; Laskowitz et al., 2017; Tu et al., 2017). A phase 1 randomized, double-blind, placebo-controlled clinical trial of CN-105 showed the peptide was safe and well tolerated in both escalating and multiple dosing regimens, among 48 healthy volunteers (Guptill et al., 2016). Of note, the plasma half-life of the peptide was determined to be 3.6 hours in these patients, significantly longer than that observed for other mimetics. Whether CN-105 provides benefit in AD has not been studied in animals or humans.

A novel apoE mimetic, COG1410 (derived from amino acids 138-149 of the apoE receptor-binding region), has been shown to improve cognitive function while reducing amyloid immunoreactivity and microglial activation after subarachnoid hemorrhage and traumatic brain injury (TBI) in mice (Gao et al., 2006; Hoane et al., 2009; Jiang and Brody 2012). This peptide crosses the BBB, and has been shown to exert its neuroprotective effects by reducing apoptosis and neuroinflammation (Wu et al., 2016), and by mitigating disruption of the BBB following experimentally-induced TBI (Pang et

al., 2017). COG1410 has also been shown to improve glucose uptake, reduce cerebral edema, and protect against neuronal atrophy in a model of TBI (Qin et al., 2017). Vitek et al. examined the effects of this apoE mimetic in a mouse model of AD, and found that the peptide ameliorates behavioral deficits and reduces plaques and tangles (Vitek et al., 2012). COG1410 was recently shown to upregulate autophagy via phosphorylation of GSK3β (Li et al., 2018). Given the fact that GSK3β phosphorylates tau, studies to determine the role of COG1410 in mouse models of tauopathy, especially those co-expressing amyloid pathology, will be of critical importance.

Another well-studied apoE mimetic, COG112, was derived from amino acids 133-149 of the receptor-binding region of apoE, and has been shown to be anti-inflammatory in mouse models of neurological disease (Laskowitz et al., 2001; Lynch et al., 2003; Li et al., 2006; Wei et al., 2013). Wang et al. described the ability of this peptide to mitigate elevation in brain amyloid levels following TBI (Wang et al., 2007). Recently, COG112 was shown to rescue BBB function following traumatic spinal cord injury in apoE-knockout mice (Cheng et al., 2018). The potential of COG112 in AD was tested by Ghosal and colleagues, who found that the peptide reduced neuro-inflammation and protected against impairment of neurogenesis and tau pathology in an AD mouse model (Ghosal et al. 2013).

An interesting mimetic is Ac-hE-18A-NH2, which is a dual-peptide comprised of residues 141-150 of the receptor-binding region of apoE covalently linked to the apoA-I mimetic 18A (Xie et al., 2012). This peptide has been shown to be anti-oxidative, and *vasculoprotective in vitro* and *in vivo* (Ramprasad et al., 2002; Gupta et al., 2005; Xie et al., 2012). The potential of this apoE/apoA-I mimetic in AD was tested in a mouse model of AD, and was demonstrated to improve cognition, decrease amyloid plaque deposition, and reduce glial activation (Handattu et al., 2013). While that study observed

enhancement of monocytic Aβ uptake by Ac-hE-18A-NH2, this peptide was recently shown to inhibit astrocyte-mediated uptake of Aβ, and to reduce Aβ-induced pro-inflammatory cytokine release (Montoliu-Gaya et al., 2018). Importantly, this dual apoE/ApoA-I peptide employs 18A as the apoA-I component. Given that the anti-atherosclerotic effect of 4F has been shown to possess superior efficacy, when compared to 18A (reviewed by Leman et al., 2013), but inferior efficacy compared to Ac-hE-18A-NH2 (Nayyar et al., 2012), it would be interesting to determine whether combining 4F with the receptor-binding region of apoE, to create Ac-hE-4F-NH2, produces more robust protective effects than Ac-hE-18A-NH2 or 4F alone in AD mouse models.

Together, these data indicate that apoE mimetics may have efficacy in a broad range of neuroinflammatory disorders, due to the increasing evidence that AD and other neurological disorders involve vascular health decline and inflammation (reviewed by de la Torre, 2002 and Zlokovic, 2004).

ApoJ Mimetics

Apolipoprotein J (apoJ), also known as clusterin, is an apolipoprotein comprised of three amphipathic and two coiled-coil α-helices, which are typical structures for molecular chaperones (Humphreys et al., 1999; Bailey et al., 2001; Ganea, 2001; Lakins et al., 2002). ApoJ has been described as a “double-edged sword” in respect to AD, showing some beneficial, and some detrimental, functions. Genome-wide association studies identified allele variants of apoJ associated with increased risk of AD, and apoJ has been suggested as a biomarker for AD (Harold et al., 2009, Lambert et al., 2009 and Song et al., 2009). Elevated apoJ expression has been observed in the hippocampi of AD patients (May et al., 1990), and elevated plasma apoJ levels were associated with

the rate of cognitive decline in a mouse model of AD (Thambisetty et al., 2010). However, genetic knockout of apoJ was associated with earlier onset and elevated levels of amyloid pathology in AD mice (DeMattos et al., 2004). A recent study showed that apoJ KO animals had significantly lower amyloid burden within the brain parenchyma, but elevated deposition in cerebral vessels (Wojtas et al., 2017). Interestingly, despite an increase in cerebral amyloid angiopathy (CAA), these animals had fewer microhemorrhages and reduced gliosis than their apoJ-competent counterparts, complicating the therapeutic potential of targeting apoJ for AD. ApoJ has been shown to bind Aβ, both *in vitro* and *in vivo* (Ghiso et al., 1993), and to reduce Aβ aggregation (Wilson et al., 2008). ApoJ has been shown to help eliminate pathogenic Aβ species across the BBB (Bell et al., 2007), although another study found that, *in vitro*, apoJ inhibits Aβ clearance mediated by primary human glial cells (Mulder et al. 2014).

The potential of apoJ mimetics to mimic the beneficial aspects of full-length apoJ has been studied. Navab et al. tested a 10 amino acid mimetic peptide derived from residues 113-122 of the full-length apoJ protein (termed apoJ[113-122]). The authors discovered that this mimetic did not possess an alpha helical structure in the absence of lipids, as 4F and other HDL mimetics do. Nevertheless, apoJ[113-122] reduced atherosclerosis in apoE-null mice, and improved HDL anti-inflammatory properties in monkeys as well (Navab et al. 2005). Importantly, D-apoJ[113-122] had a much longer half-life in the plasma than D-4F, and retained its atheroprotective effects for up to 48 hours, after a single dose. ApoJ[113-122] has also been shown to reduce inflammation and chemotaxis of primary monocytes derived from patients with systemic lupus erythematosus (Skaggs et al., 2010). Recently, apoJ[113-122] was found to reduce fat accumulation and weight gain in LDLR-knockout mice given an atherogenic diet (Rivas-Urbina et al., 2017)

The potential of apoJ mimetics in AD has not yet been described. However, apoJ[113-122] was recently shown to inhibit the uptake of Aβ by astrocytes, and the peptide did not reduce Aβ-induced IL-6 release, unlike an apoE mimetic (Montoliu-Gaya et al., 2018). The authors argue that reduced Aβ uptake by astrocytes protects their ability to continue supporting neuronal health. Interestingly, the authors' earlier work indicates that apoJ[113-122] promotes the assembly of Aβ into a less-pathogenic aggregation state, which they term a "worm-like pathway" (Montoliu-Gaya et al., 2017). Recent work from our own laboratory, in unpublished data, show that daily i.p. administration of D-apoJ[113-122] for 3 months induced a dramatic decrease in amyloid pathology and rescued memory deficits in the APP/PS1 mouse model of AD (Hottman et al., *In Preparation*).

Recombinant HDL

Another method of mimicking the beneficial functions of HDL is the production of recombinant HDL (rHDL) by the combination of a purified apolipoprotein, or mimetic peptide, and lipids. The use of full-length apoA-I-containing rHDL reduces both acute and chronic vascular inflammation and oxidative stress in rabbits (Tabet et al., 2010; Patel et al., 2010; Di Bartolo et al., 2011). rHDL utilizing human apoA-I harboring a mutation associated with reduced risk of atherosclerosis has been shown to be anti-inflammatory and atheroprotective in humans (Franceschini et al., 1980; Nissen et al., 2003).

Further, a phase 2 clinical trial using CER-001, an apoA-I-containing recombinant HDL particle (Tardy et al., 2014), showed improvements in carotid artery wall thickness when administered to patients already receiving maximal LDL-lowering therapy (Hovingh et al., 2015). However, a prospective, double-blinded, randomized trial of CER-001 infusion in 507 patients with atherosclerosis showed no benefit of the particle in reducing atheroma

volume over a period of 6 weeks (Tardif et al., 2014), despite earlier clinical data indicating that CER-001 targets atherosclerotic plaques, increases cholesterol efflux, and reduces carotid artery wall inflammation and thickness in human patients (Kootte et al., 2015; Zheng et al., 2016). Whether longer treatment, oral administration, or treatment of a different patient population might influence the efficacy of this recombinant HDL particle will require further studies.

As discussed earlier, however, the use of full-length apoA-I presents significant problems in terms of mass-production and therapeutic delivery route. An interesting study sought to generate rHDL by using a flexible apoA-I mimetic, designed by attaching three 23mers derived from amino acids 221-241 of the 10^{th} helix of apoA-I to a scaffold, and then adding lipids to convert the construct into an HDL-like nanoparticle. The authors observed a pronounced reduction of plasma LDL-cholesterol, an effect not observed with most other apoA-I mimetics that have been studied (Zhao et al., 2014). These constructs were strongly anti-atherogenic when administered either i.p. or orally, despite the peptide being comprised entirely of L-amino acids and undetectable in the plasma. This observation may be further evidence to support the idea that enteric involvement of apoA-I mimetic peptides is key to their efficacy, at least in regards to atherosclerosis. Another study created rHDL using the bi-helical apoA-I mimetic 37pA alongside palmitoyl-oleoyl-phosphatidylcholine, finding that the synthetic HDL preparation was anti-inflammatory and cardioprotective (Gomaraschi et al., 2008).
In regards to effects relevant to AD, a recent study found that a single intravenous injection of reconstituted HDL containing apoA-I and soy phosphatidylcholine was associated with reduced amyloid levels in APP/PS1 mice 24 hours later, while no differences were observed after four consecutive once-weekly injections (Robert et al. 2016). In addition, four-week administration of reconstituted HDL particles containing

lipidated apoE3 increased Aβ clearance and improved cognitive function in AD mice (Song et al. 2014). The therapeutic potential of recombinant HDL-like particles in human neurological disease is an intriguing possibility, one which should be addressed by clinical studies.

Summary

The accumulated evidence suggests a strong potential for HDL-mimetic peptides in protecting against AD. Drawing from the well-established connections between CVD risk and AD described earlier in this dissertation, the dramatic effects of these peptides in mitigating atherosclerosis and reducing inflammation are promising not only in systemic vascular health, but in the brain as well.

Despite the significant advantages conferred by the use of small mimetic peptides in lieu of their parent full-length proteins, these agents are not without their own pitfalls. Low bioavailability, short half-lives, and the risk of antibody response are potential drawbacks to this therapeutic avenue. One report suggests that the use of an ELISA specifically designed to identify preβ_1 HDL, a marker for RCT, could aid the development of HDL-mimetic small-molecules (Troutt et al., 2008), which would eliminate many of the previously mentioned drawbacks of mimetic peptides. Whether this assay would produce molecules relevant to AD pathology has not been explored.

Although somewhat more limited than that of atherosclerosis, significant and growing evidence suggests beneficial functions of these peptides in AD as well. Further studies of the precise mechanisms by which these peptides may enact beneficial effects in the context of AD are needed. Given the proven clinical safety of the 4F peptide, in particular, a clinical trial to determine whether 4F improves cognition or delays decline in patients with MCI or AD would be justified.

Interestingly, 4F and other apoA-I mimetic peptides have been shown to inhibit tumor formation and progression in mice (Cedó et al., 2016; Peng et al., 2017; Chattopadhyay et al., 2018). In addition, 4F has been shown to mitigate inflammation associated with viral and bacterial infection (Van Lenten et al., 2004; Moreira et al., 2014; Sharifov et al., 2014). 4F was also shown to restore the anti-inflammatory and anti-oxidative properties of HDL from human immunodeficiency virus (HIV-1) patients. An estimated 50% of HIV-1 cases have dementia, despite the advent of highly active antiretroviral therapy (HAART) (Saylor et al., 2016). Recently the potential role of a wide range of systemic infections in AD risk and pathogenesis has also gained attention (Maheshwari et al., 2015; Licastro et al., 2016, Eimer et al 2018). Due to the increased risk of both cancer and infection with aging, the patient populations that are likely to benefit from HDL-mimetic peptides in relation to cognition may also benefit from these other observed effects of 4F. These multi-faceted protective effects of 4F may also mitigate the compounding effects of systemic and neuroinflammation driven by infection, cancer, and/or early-stage AD, thereby delaying cognitive decline in affected patient populations. Whether that is the case remains to be studied experimentally.

Systemic Versus Brain ApoE in Alzheimer's Disease

ApoE is a 34 kDa glycoprotein that is incorporated into lipoprotein particles in the plasma (Mahley et al, 1988), as well as the CSF and brain parenchyma (Comley et al., 2011). The canonical function of apoE is in transporting cholesterol and other lipids, mediated by cell surface apoE receptors (Mahley, 2017; Kanekiyo et al., 2014), although apoE also plays important roles in immunomodulation, synaptic plasticity, signal transduction, and proteostasis (Holtzman, 2012).

The apoE gene is polymorphic in humans, consisting of three alleles (ε2, ε3, and ε4), which leads to the production of three distinct isoforms of apoE protein: apoE2, apoE3, and apoE4. The three apoE isoforms differ at positions 112 and 158: apoE2 has Cys residues at both positions, apoE3 has a Cys residue at 112 and an Arg residue at 158, and apoE4 has Arg residues at both positions (Weisgraber et al., 1981; Rall, 1982). These differences alter the structure and physiological function of apoE. ApoE binds primarily to the LDL receptor family, which is the receptor mostly responsible for cholesterol homeostasis. ApoE3 and apoE2 are more selective to HDL, while apoE4 preferentially binds to triglyceride-rich, very low density lipoproteins (VLDL), leading to downregulation of LDL receptors and thus reduced clearance of LDL and increased plasma cholesterol levels (Mahley, 2016). These differences in affinity lead to increased risk of atherosclerosis and stroke among apoE4 carriers, as well as increased risk of type III hyperlipoproteinemia in those carrying apoE2 (Mahley, 2016).

Interest in apoE in the central nervous system (CNS) increased greatly when it was discovered that the apoE ε4 allele is the strongest genetic risk factor for late-onset AD, while apoE2 protects against the development of AD (Corder et al., 1993, Saunders et al. 1993, Strittmatter et al., 1993). The allele frequency of apoE ε4 is approximately 15% in the general population, however, it is enriched to ~ 40% in AD patients. ApoE ε3, the most common allele, has a frequency of approximately 77%, and is considered to be neutral in regards to neurodegeneration, while apoE ε2 allele is less common in the general population (~ 8% allele frequency) (Corder et al., 1993; Saunders et al., 1993; Strittmatter et al., 1993).

Cholesterol homeostasis is integral to normal brain function. While the brain comprises approximately 2% of the total body weight, as much as 25% of all cholesterol in the body is found in the brain (Dietschy and Turley, 2001). Circulating lipoproteins are unable to

cross the blood brain barrier, however, small HDL particles can enter the brain, stimulating interest as to whether plasma HDL levels/function influence brain cholesterol homeostasis and function (Hottman, 2014; Vitali et al., 2014). ApoE genotype, cardiovascular disease, and AD are mechanistically linked by cholesterol homeostasis and related pathways, raising the question as to how these two carefully separated biologic systems influence one another in health and disease. This brief review attempts to capture recent findings on the role of both systemic and brain apoE in the pathogenesis of AD, and their implications for the development of apoE-targeted therapeutics.

Systemic versus brain apoE

Perhaps the most basic support for two distinct pools of apoE in the human body is the fact that while apoE is produced both in the periphery and in the brain (Panza et al., 2003), the protein does not cross the blood brain barrier (BBB) (Linton et al., 1991). In the periphery, apoE is primarily produced by the liver (Panza et al., 2003), however, it is also expressed by macrophages (Mahley, 1988). In the brain, the primary source of apoE is astrocytes, while microglia, oligodendrocytes, and damaged neurons may also produce the protein (Boyles et al., 1985; Uchihara et al., 1995; Xu et al., 2006; Zhang et al., 2014). Concentrations of apoE differ highly between the two compartments; apoE levels are around 50ug/mL in the plasma, whereas they are roughly 5-10ug/mL in the CSF (Panza et al., 2003).

The role of apoE in response to pathological insults is also tissue specific; when exposed to cytokines, macrophages respond by modulating apoE secretion, while cytokine exposure in hepatocytes does not influence apoE secretion (Kockx et al., 2018). In the brain, microglia upregulate apoE in response to inflammatory stimuli, and it

has been suggested that neurons may express apoE as well, when under stress (Xu et al., 2006; Wang et al., 2018).

Homozygous apoE2 binds defectively to LDL receptors, causing type III hyperlipidemia. Some studies indicate that this disease only occurs in the presence of other conditions such as diabetes, hypothyroidism, and obesity, which leads to fewer LDL receptors, limiting the ability of apoE2 to modulate cholesterol clearance (Mahley, 2017). ApoE4 has a binding preference with VLDL resulting in elevated plasma LDL levels, and an increase risk of atherosclerosis (Mahley, 2016). As VLDL is expressed in the presence of apoE4, clearance of these particles from the plasma is accelerated in the liver, resulting in fewer LDL receptors, and an increase in plasma LDL. Evidence also indicates that apoE genotype may influence other key apolipoproteins in the periphery and in the brain. ApoE4 targeted-replacement mice, when compared to age-matched apoE3 counterparts, had lower levels of apoA-I in the plasma, but elevated levels in the brain (Boehm-Cagan et al., 2016). In this study, apoJ levels were found to be higher in the plasma of apoE4 animals as well.

A recent study of human apoE3 and apoE4 carriers provided further evidence of distinct peripheral and central apoE pools in the context of AD. The study demonstrated that amyloid deposition was associated with elevated levels of apoE3, but not apoE4, and this resulted in an increased apoE4/apoE3 isoform ratio in heterozygote individuals in the CNS, while the ratio decreased in the periphery (Baker-Nigh et al., 2016). Further, apoE levels in the CSF were associated with CSF Aβ levels, whereas plasma apoE levels lacked this correlation.

Role of peripheral and central apoE pools in cognition and AD

Peripheral apoE

Several lines of evidence support the importance of plasma apoE in cognition. Mice genetically engineered to express apoE in the periphery, but not in the brain, had improved cognitive performance and a preserved AMPA/NMDA ratio (Lane-Donovan et al., 2016). However, this study also highlights the importance of apoE in the brain, showing that brain apoE KO animals still had synaptic loss and dysfunction, despite their attenuated learning deficits. These data indicate that apoE, even that in the periphery, can influence the deleterious outcomes of synapse loss, which is known to be the best predictor of cognitive impairment in humans with AD. However, the continued presence of synaptic loss and dysfunction after restoration of plasma apoE indicates that apoE in the brain also plays an important role in maintaining neuronal health.

Another study, from the laboratory of Dr. Guojun Bu, adds evidence to support the importance of peripheral apoE in the context of neurological health. Bu and colleagues found that peripheral apoE4 was associated with increased loss of grey matter volume in the posterior cingulate, and reduced glucose metabolism in the hippocampus (Nielsen et al., 2017). Interestingly, in this cohort of 128 cognitively normal apoE3/apoE4 carriers, females with higher plasma apoE3 levels performed better on the verbal reasoning (similarities) subtest of the Wechsler Adult Intelligence Scale test.

The role of apoE in the periphery is not confined to proteins in the plasma, however. It has been shown that in young healthy patients carrying the apoE4 allele, circulating peripheral lymphocytes express elevated levels of GSK3β, and phosphorylated tau (Badia et al., 2013). interestingly, these healthy young individuals experienced subjective cognitive impairment, when compared to age-matched apoE3 carrier controls. These data highlight the role of peripheral apoE in driving early pathological hallmarks of AD.

ApoE genotype modifies immune response to acute and chronic inflammation. Systemic inflammation is a well-established risk factor for AD, while neuroinflammation is a key pathological hallmark of the disease. Metabolic disorders including obesity, diabetes, dyslipidemia, and high blood pressure are all associated with both chronic systemic inflammation and risk of dementia (Alberti et al., 2006; St-Onge et al., 2009; Farooqui et al., 2011; van Himbergen et al., 2012; Nation et al., 2015; Arvanitakis et al., 2018).

ApoE in the CNS

ApoE has long been known to be a key regulator of amyloid pathology in the brain, and recent evidence highlights the role of apoE4 in driving early pathological accumulation of Aβ in the AD brain (Liu et al., 2017). Further, a recent report confirms previous studies showing that mice lacking apoE have reduced Aβ load (Ulrich et al., 2018). However, this study indicates that plaques in the apoE-deficient mice are less compacted, have more plaque-associated dystrophic neurites, fewer plaque-associated microglia, and reduced microglial activation than their apoE-expressing counterparts. This indicates that microglial apoE is important in mediating the control of amyloid toxicity in the brain. In addition, a recent study indicates that cholesterol increases the rate of Aβ aggregation (Habchi et al., 2018). The canonical influence of apoE on cholesterol trafficking may be a driving mechanism for its early role in AD pathogenesis.

In the context of AD, apoE has been most extensively studied in relation to Aβ aggregation and clearance. ApoE is responsible for the clearance of Aβ and studies have shown that apoE4 is deficient in this function, resulting in elevated amyloid deposition in the brain (Mahley, 2017). Recent studies using induced pluripotent stem cells (iPSCs), derived from patients homozygous for either apoE3 or apoE4, offer unique insights into the role of apoE4 in human pathology. ApoE4 neurons exhibited elevated

Aβ production, tau phosphorylation, and GABAergic neuron atrophy, when compared to those expressing apoE3 (Wang et al., 2018). Highlighting the unique influence of apoE on different cell types, another similar study evaluated the role of apoE4 in neurons and glial cells. The authors found that transcriptional alterations associated with apoE4 were most commonly related to synaptic function in neurons, lipid metabolism in astrocytes, and immune activity in microglia (Ling et al., 2018). At the same time as apoE4 neurons released greater levels of Aβ into the culture medium, microglia and astrocytes failed to clear the toxic peptides away as effectively as their apoE3-expressing counterparts. The use of iPSCs from human patients is a significant advance in the field of AD research, which has been plagued by incomplete and clinically irrelevant model systems for decades. Further, the use of iPSCs, induced to take on peripheral cell phenotypes, may improve our understanding of the role of systemic apoE in driving AD risk and pathological progression, as well.

The role of apoE lipidation state in the context of AD pathogenesis has gained significant attention in recent years. Lipidated apoE facilitates the removal of Aβ peptides, while lipid-poor apoE stimulates the formation of amyloid plaques (Kockx et al., 2018). ApoE4 lipidation is decreased in the brain, when compared with other isoforms (Mahley, 2017). Importantly, a recent study highlighted the importance of lipidated apoE in driving amyloid pathology, as treatment with an antibody recognizing only non-lipidated apoE reduced Aβ load in the brains of AD mice (Liao et al., 2018)

An important recent advance in the field of AD research was the discovery that apoE and TREM2 are binding partners (Atagi et al., 2015; Bailey et al., 2015; Yeh et al., 2016). Polymorphisms of TREM2 were associated with increased risk of AD in recent GWAS studies (Guerreiro et al., 2013; Jonsson et al., 2013; Wang et al., 2015). While the majority of research on TREM2 has focused on microglia in the brain, but is also

expressed in the periphery by mononuclear cells, where its expression is upregulated amongst MCI patients who later progress to AD (Casati et al., 2018). The impact of the apoE-TREM2 interaction on AD risk and pathological progression has not yet been determined.

Another pivotal recent discovery from the laboratory of Dr. David Holtzman showed that apoE4 is also involved in the pathogenic progression of tau-mediated toxicity and neuroinflammation (Shi et al., 2017). As tau pathology is a driving factor in AD, vascular dementia, post-stroke dementia, and vascular cognitive impairment (Akinyemi et al., 2017; Kim et al., 2018), the role of apoE in modifying tau pathogenesis has significant clinical implications. Further, amongst participants with AD and vascular dementia, apoE4 carriers were found to have a higher burden of both amyloid and tau immunoreactivity than non-carriers (Akinyemi et al., 2017). Whether peripheral apoE pools mediate these observations has not been studied.

ApoE at the blood brain barrier

While apoE exists in two distinct pools in the human body, the influence of each pool on the other, and on AD pathogenesis, remains an important concern. The most obvious place for the differential influences to converge is at the junction of the periphery and the central nervous system, the blood brain barrier (BBB). The importance of apoE at the BBB is highlighted by the fact that most cells participating in the formation and maintenance of this complex biological barrier, including astrocytes, pericytes, endothelial cells, smooth muscle cells, and neurons (when stressed), express apoE and/or its receptors (Kanekiyo et al., 2014). Other cells known to interact with the BBB, such as macrophages and microglia, also express apoE.

It has been known for some time that cerebrovascular damage is a hallmark of AD. Approximately 80% of all AD patients have cerebral deposition of Aβ, known as cerebral amyloid angiopathy (CAA), which is associated with disruption of the BBB (Serrano-Pozo et al., 2011). This dysfunction of the cerebral vasculature has recently re-entered the spotlight in the AD field (Love and Miners, 2016), and the role of apoE in this process is an area of great interest. Early studies indicated that apoE deficiency leads to damage of the BBB (Methia et al., 2001; Fullerton et al., 2001; Hafezi-Moghadam et al., 2007). More recently, it has been shown that the influence of apoE on BBB integrity, as well as cerebral blood flow, is isoform-dependent (Nishitsuji et al., 2011; Bell et al., 2012). In apoE4-expressing 5xFAD mice, it was recently demonstrated that peripheral inflammation induced by LPS injection resulted in reduced cerebral vessel coverage, leakiness of the BBB, and elevated CAA (Marottoli et al., 2017). In line with these results, additional studies indicate that apoE4 increases the prevalence and severity of CAA in humans (Ringman et al., 2014; Esiri et al., 2015). These data indicate that Aβ, apoE4 status, and peripheral inflammation - all known to induce vascular damage in their own right - may act synergistically to wreak havoc on cerebral blood vessels and the BBB.

An important paper from the laboratory of Dr. Cheryl Wellington demonstrated the development of a 3D model of CAA using human primary endothelial cells, smooth muscle cells and astrocytes attached to a scaffold-directed, dynamic, pulsatile flow bioreactor system (Robert et al., 2017). Using this model Dr. Wellington's group showed that while circulating apoE (representing that from the peripheral pool) in general facilitated the clearance of Aβ across the synthetic BBB, apoE4 was deficient in this capability compared to apoE2. Further, transport of $A\beta_{42}$ was more robust than that of

$A\beta_{40}$, consistent with the fact that $A\beta_{40}$ is more prone to becoming stuck in the cerebral vasculature *in vivo*, as the primary constituent of CAA (Serrano-Pozo et al., 2011).

While the publication of the data has not yet occurred, a recent poster from the laboratory of Guojun Bu, presented at the Advances in Neurodegenerative Disease Research and Therapy / New Frontiers in Neuroinflammation Keystone Joint Symposia in July of 2018, highlighted the importance of peripheral apoE in respect to BBB integrity and cognition (https://www.alzforum.org/news/research-news/apoe-has-hand-alzheimers-beyond-av-beyond-brain). The authors induced expression apoE, only in hepatocytes, on an apoE KO background, in order to study the effect of peripheral apoE4 on the brain. They found that peripherally-expressed apoE4 was associated with increased BBB leakiness, elevated neuritic dystrophy, and impairment in cognitive performance.

Implications for treatment of mild cognitive impairment and dementia

While Aβ-targeted therapeutics, including anti-amyloid antibodies and BACE1-inhibitors, continue to disappoint in clinical trials, apoE becomes an ever more attractive therapeutic target in AD. In order for an apoE-targeted treatment to be effective, however, it will be important to understand the differential and cooperative roles that the peripheral and central apoE pools play in AD pathogenesis.

One example of this is apoE-targeted antibodies, which have recently been shown to reduce amyloid accumulation in the brains of mice (Liao et al., 2014; Liao et al., 2018). Similar results were obtained with both a central and peripheral delivery of the antibody. This raises the question of which pool of apoE, or both, is necessary to achieve the drug effect. Studies of these antibodies using mice expressing apoE only in the periphery (or only in the brain), would help to answer this question.

In 2016, researchers led by Dr. Leon Tai found that female EFAD mice expressing apoE4 are cognitively impaired, and have damage at the BBB as well as reduced vessel coverage in the brain. Highlighting the impact of the vasculature in driving these phenomena, as well as the links between apoE4, vascular dysfunction, and cognitive impairment, EFAD mice expressing apoE4 that were peripherally administered epidermal growth factor (EGF) were spared from the cognitive and vascular deficits observed in untreated animals (Thomas et al., 2016; Thomas et al., 2017).

As discussed earlier, many groups have focused efforts on drugs targeting the nuclear receptors (LXRs and RXRs) as potential therapeutics in AD. These agents have been demonstrated to upregulate ABCA1 and apoE, increase apoE lipidation, and to reduce amyloid pathology and cognitive impairment in mouse models of AD (Moutinho et al., 2017). The most publicized of these agents is bexarotene, owing to its status as an FDA approved agent for the treatment of T-cell lymphoma. Bexarotene was shown to mitigate AD-related pathology and memory deficits in animal models, although some controversy around the anti-amyloid effects and safety of this agent have arisen (Tesseur et al., 2013; Veeraraghavalu et al., 2013). Further complicating the story, a small placebo-controlled phase 2 clinical trial of bexarotene showed no alteration in Aβ levels in AD patients. However, subgroup analysis determined that apoE4-carriers were holding back the overall analysis, as those carrying apoE2 and apoE3 did in fact have reductions in amyloid burden (Cummings et al., 2016). This is in contrast to studies in animal models, where bexarotene was shown to mitigate apoE4-driven pathology (Boehm-Cagan et al., 2014; Mounier et al., 2015).

As discussed in detail earlier, several groups have studied the potential of HDL-mimetic peptides and recombinant HDL in the treatment of AD. These HDL mimetics are anti-inflammatory and anti-oxidative, and several have been shown to be safe and well-

tolerated in human clinical trials (Bloedon et al., 2008; Dunbar et al., 2017). A number of these peptides, derived from or based upon key receptor-binding regions of apoA-I, apoE, or apoJ, have been shown to improve cognition, reduce neuronal damage, and to mitigate AD-related pathology in animal models (Handattu et al., 2009; Vitek et al., 2012; Handattu et al., 2013; Boehm-Cagan et al., 2016; Laskowitz et al., 2017). Recent work in our own laboratory, described in chapter 2 of this dissertation, indicates that HDL-mimetic peptides act to alleviate apoE lipidation deficiency and counteract Aβ-induced lipidation deficiency in glial cells (Chernick et al., 2018). Further, chapter 3 of this dissertation details our studies demonstrating that these peptides can rescue apoE4-related lipidation deficiency and reduce amyloid pathology and learning deficits in AD mice.

Another line of evidence indicates that apoE4, expressed in neurons under stress conditions, is cleaved into neurotoxic fragments. A recent report from Yadong Huang's group shows that small-molecule structure-correctors, designed to restore apoE4 to an apoE3-like tertiary structure, mitigates this detrimental effect of apoE4 in neurons derived from human iPSCs (Wang et al., 2018).[42] For small molecules that can cross the BBB more readily than antibodies, it will still be important to understand how these drugs impact peripheral apoE levels and function.

Summary

The current evidence highlights the far-reaching impact of apoE in AD. From peripheral tissues to neurons, and nearly all relevant pathways in between, apoE plays a pivotal role, and apoE4 drives pathology. With a continued need for effective therapeutics to halt the clinical progression of AD, and a series of recent disappointing results from highly anticipated clinical trials, apoE has rapidly risen to the forefront as a druggable

target. It will be important to understand how apoE-targeted therapeutics modify potential comorbidities, including cardiovascular disease and systemic inflammation, as well as any potential adverse events associated with systemic administration of these agents. While central pools of apoE drive key pathological hallmarks of AD, including amyloid deposition, tau hyperphosphorylation, and neuroinflammation, peripheral apoE contributes to systemic inflammation, amyloid clearance, and BBB integrity. In both the periphery and in the brain, apoE4 is a key driver of pathological progression. Many questions remain unanswered as to the direct and indirect impact of peripheral apoE on cognition and AD, and a focus on this area will be vital to continued efforts to eradicate this horrendous disease.

Cigarette Smoking, Cardiovascular disease, and Alzheimer's Risk

Around the world, 2 billion people are estimated to smoke cigarettes (DeMarini, 2004). Prevention efforts and quitting aid have reduced the prevalence of cigarette use in the United States, however, there remain an estimated 44 million smokers (Dube et al., 2009), a number that is thought to be underestimated (DeInevo et al., 2008). Cigarette smoking accounts for 1 out of every 5 deaths in America, and smoking-related healthcare costs and lost productivity amount to a nearly $200 billion deficit, every year (Dube et al., 2004; Ceners for Disease Control and Prevention, 2011). Despite decades of awareness efforts, the ratio of individuals who have quit smoking to those who have never smoked (known as the quit ratio) has remained unchanged since 1998 (Alpert et al., 2013).

According to the American Heart Association, cigarette smoking is the preeminent preventable cause of cardiovascular disease (American Heart Association, 2014). Current and past smokers are at a greater risk of atherosclerosis, coronary heart

disease (CHD), hypertension, myocardial infarction (MI), and stroke (Wolf et al., 1988; US Dept of Health and Human Services, 1989). Importantly, smoking is also known to compound with other CVD risk factors (Anderson et al., 1991). Cessation of smoking has been shown to drastically reduce the risk of CHD and stroke (Wolf et al., 1988; Ockene et al., 1990). Due to the relationship between CVD and AD, as discussed above, the relevant question is whether smoking is also associated with increased risk of AD.

Cigarette Smoking and AD Risk

Although some conflict exists in the literature, the overall consensus is that cigarette smoking is associated with elevated risk of developing AD (Durazzo et al., 2014). Interestingly, the majority of studies finding no association between smoking and AD risk were funded by tobacco companies, while most studies funded by other sources report a significant increase in AD risk among smokers (Cataldo et al., 2010). Given the tobacco industry's long-standing stance in opposition to scientific consensus, and nefarious practices in attempting to influence that consensus, the results obtained by studies funded by tobacco companies are of dubious credibility and should be interpreted with caution (Milberger et al., 2006; Cataldo et al., 2010; Brandt, 2012). Alarmingly, a report from the World Health Organization (WHO) highlights the lengths to which the tobacco industry has gone, and continues to go to, in order to confuse, impede, and manipulate scientific efforts to study smoking and smoking-related illness (WHO, 2008). The WHO report describes these reprehensive activities, stating, "the history of tobacco industry involvement in research has shown that the results are often manipulated, suppressed, or used incorrectly by non-scientists to suit the needs of the tobacco industry." This review will therefore consider any and all studies conducted by, on the behalf of, or with support from tobacco companies to be invalid and excluded them from further discussion.

Several studies compared the relative exposure to cigarettes, measured in pack-years (the number of cigarette packs a person smokes per day multiplied by the number of years they have smoked), finding elevated risk of developing AD amongst active and former-smokers, regardless of their exposure (Ott et al., 1998). Another two studies with similar designs, combining both active and former-smokers into a single group, found an increased risk of AD in those with 27-56 pack-years of exposure, when compared to participants with < 27 pack-years. Interestingly, smokers with > 56 pack years showed no change in AD risk, which the authors contend is due to survivor bias (Tyas et al., 2003; Juan et al., 2004). An effect of exposure was observed in another study, which showed that active-smokers with > 20 pack-years had a nearly 2-fold greater risk of AD than those with < 20 pack-years (Reitz et al., 2005). Further, a large American cohort study found that smoking more than 2 packs of cigarettes per day was associated with increased risk of developing AD (Rusanen et al., 2010).

The data concerning the influence of quitting on AD risk are more nuanced. While two reports found that former-smokers did not have an increased risk of AD, compared with never-smokers (Merchant et al., 1999; Reitz et al., 2005), another study found that former-smokers who were male, but not female, had a 2-fold increased risk for AD, when compared to never-smokers (Launer et al., 1999). AD onset occurred significantly earlier in both current smokers and those who had quit, when compared with never-smokers (Ott et al., 1998; Merchant et al., 1999; Sabbagh et al., 2005). Mortality was also found to increase alongside cigarette exposure, amongst both controls and AD patients (Tyas et al., 2003). It has been proposed that smoking accounts for nearly 600,000 (11%) of AD cases in the US and almost 5 million (14%) cases around the world (Barnes and Yaffe, 2011). The authors of this report contend that reducing the total number of smokers in the US and around the world by 10% would decrease AD prevalence by 51,000 and

412,000 cases, respectively. In support of this idea, a recent study in China, where cigarette smoking prevalence has been and remains extremely high (Liu et al., 2016), smoking cessation was associated with reduced risk of dementia (Deng et al., 2018). This highlights the importance of continued awareness and education, as well as efforts to aid those attempting to quit.

Mixed results have been obtained in regards to the role of apoE genotype in modifying the risk of AD amongst smokers. In a large cohort study performed in Finland, an increased risk of AD was found amongst apoE4 carriers who had smoked during midlife (Rusanen et al., 2010). In another study, former-smokers carrying an apoE4 allele were found to have a significantly lower risk of AD than never-smokers (Aggarwal et al., 2006). However, this study was performed in an urban biracial cohort, which begs the question of whether this apoE4-mediated effect was due to survivor bias, based upon the known elevated risk of cardiovascular disease among apoE4 carriers, urban communities, and black Americans (Kulshreshtha et al., 2014). These three factors, together, may have greatly reduced the likelihood that apoE4-carrying former-smokers reached an age at which AD could present clinically. In both case-controlled and cohort studies, survivor bias may lead to significant underestimation of AD risk associated with smoking (Kukull, 2001; Debanne et al., 2007; Hernan et al., 2008; Chang et al., 2012; Weuve et al., 2012). Premature death attributed to smoking-related illness reduces the number of smokers who may have gone on to develop AD had they lived long enough, while those who do survive are biased toward being healthier (Chang et al., 2012). Taken together these data imply that smokers who carry an apoE4 allele are at a greater risk of developing AD, although further studies are needed.

Smoking and AD pathological hallmarks

Several lines of evidence suggest that cigarette smoke is associated with elevated AD pathology. A study in neuroblastoma cells expressing human APP harboring the Swedish mutation found that *in vitro* administration of cigarette smoke condensate was associated with elevated production of Aβ (Giunta et al., 2012). Animal studies provide further evidence of this detrimental effect of cigarette smoke. Rats exposed to cigarette smoke were found to have elevated levels of Aβ and phosphorylated tau in the hippocampus (Ho et al., 2012), while another study showed a dose-dependent effect of cigarette smoke in promoting increased hippocampal and cortical Aβ deposition, neuritic plaques, gliosis, and hyperphosphorylated tau (Moreno-Gonzalez et al., 2013). In humans, florbetapir retention (an imaging marker for fibrillar amyloid load in the brain), showed that former and current smokers had elevated Aβ deposition, when compared with never-smokers (Durazzo et al., 2014). Human autopsy data support these findings. Current smokers and those who had quit smoking showed elevated levels of Aβ deposition in the form of neuritic plaques, although no differences in neurofibrillary tangles were observed (Tyas et al., 2003). In the cases included in that study, the risk of AD, defined by the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) neurological battery scores, was more than twice as high among current and former smokers, when compared with never-smokers. Some studies contradict these findings (Ulrich et al., 1997; Perry et al., 2000; Hellstrom-Lingahl et al., 2004; Sabbagh et al., 2005), however, small sample sizes, inconsistent definition of smoking status, and failure to replicate well-established findings from previous studies (i.e. apoE4 genotype was associated with reduced amyloid burden) call into question the robustness of these reports.

The influence of nicotine in the absence of smoking on pathological hallmarks of AD has been studied to some extent. Data indicates that nicotine administration to

neuroblastoma cells results in elevated phospho-tau levels, and Aβ is known to bind α7-nicotinic acetylcholine receptors (nAChRs) (Hellstrom-Lindahl et al., 2000; Oz et al., 2013). Interestingly, studies in animals indicate that nicotine, administered by alternate routes than inhalation, resulted in reduced pathological hallmarks of AD, with some caveats. Subcutaneous injection of nicotine was found to reduce soluble APP fragments and elevate sAPP levels in the CSF of rats (Lahiri et al., 2002; Utsuki et al., 2002). and also to reduce Aβ and β-secretase levels in the hippocampus following intraventricular injection of amyloid peptides (Srivareerat et al., 2011). These animals also showed improved memory and elevated synaptic plasticity (long-term potentiation; LTP), when compared to animals that received intraventricular amyloid alone. In the commonly used mouse model of AD expressing the Swedish mutation, nicotine deliver via the drinking water was associated with reduced neuritic plaques and insoluble Aβ levels (Nordberg et al., 2002). These data were further confirmed by a study using the APP/PS1 mouse model of AD, in which oral administration of the primary metabolite of nicotine, cotinine, was found to reduce insoluble Aβ levels in the cortex and hippocampus, and to reduce Aβ oligomerization *in vitro* (Escheverria et al., 2011). However, another study using the 3xTg model of AD (which includes the expression of the human P301L tau mutation alongside the APP/PS1 system, allowing for the modeling of tau pathology, which is commonly lacking in mouse models of AD) failed to replicate the earlier findings of reduced insoluble Aβ in mice administered nicotine in the drinking water (Oddo et al., 2005). Importantly, the authors observed elevated phospho-tau levels and aggregation in the hippocampus of nicotine-treated animals, an effect dependent upon age-related decline in α7 nAChR density. These somewhat conflicting data raise the question as to whether promoting the use of smoking alternatives may reduce or further elevate the risk of AD for nicotine users. The relative consistency of reduction in amyloid pathology and elevation tau pathology - in light of the more robust correlation between tau pathology

and cognitive decline than that of amyloid - would argue against the use of nicotine by any route of administration.

Smoking-induced oxidative stress has also been proposed as a possible mechanism by which cigarette smoking contributes to risk of AD (Durazzo et al., 2014), in part mediated by cardio- and cerebrovascular pathways (Barnes and Yaffe, 2011; van Norden et al., 2012). Oxidative stress is a highly relevant pathway in AD. Aβ is known to induce oxidative stress (Butterfield et al., 2013), and oxidative stress is itself thought to initiate AD pathogenesis, by increasing cleavage of APP by β-secretase and elevating tau hyperphosphorylation in the brain (Mondragon-Rodriguez et al., 2010). Cigarette smoke contains high concentrations of oxidizing agents that are known to promote oxidative stress and gliosis in the brain (Ambrose and Barua, 2004; Valavanidis et al., 2009; Mazzone et al., 2010).

Smoking and cognition

A significant body of evidence indicates that smoking is associated with brain imaging abnormalities and impaired cognitive function, even amongst young, healthy individuals. Poorer performance related to executive functions, processing speed, and learning and memory have been associated with cigarette smoking in persons ranging from adolescence to old age (Durazzo et al., 2010; Durazzo et al., 2012a; Wagner et al., 2012). Further, white matter volume, cortical thickness, and cortical perfusion are reduced in current smokers, when compared with those who have never used cigarettes (Brody et al., 2004; Azizian et al., 2009; Sharma and Brody, 2009; Zhang et al., 2011; Kuhn et al., 2012; Durazzo et al., 2013). Many of these abnormalities were observed in brain regions relevant to MCI and AD (De Santi et al., 2001; Langbaum et al., 2009). These cognitive impairments and imaging abnormalities were related, in some studies,

to the level of cigarette exposure (Brody et al., 2004; Gallinat et al., 2006; Kuhn et al., 2010; Durazzo et al., 2012a; Durazzo et al., 2012b). Some studies suggest that adult former-smokers had intermediate cognitive performance, when compared with current and never-smokers (Durazzo et al., 2010). Studies using computed tomography (CT) and magnetic resonance imaging (MRI) have shown that elderly current and former smokers experience increased global brain atrophy and reduced grey matter density, when compared to never-smokers (Almeida et al., 2008; Durazzo et al., 2010). As CVD is a well-established risk factor for cognitive impairment (Leritaz et al., 2011), the influence of cigarette smoking on cognition is likely mediated, at least in part, by CVD and cerebrovascular-related pathways.

Summary

Taken together, these data indicate that smoking, the greatest modifiable risk factor for CVD, is also a risk factor for AD. This elevated risk is mediated in part by the same pathways responsible for increasing risk of CVD, and is therefore highly relevant to the work presented in this dissertation. Efforts to reduce cigarette use, improve cardiovascular health, and mitigate inflammation (both in the periphery and in the brain) may be of significant benefit in preserving cognitive function among smokers.

Role of Systemic and Neuroinflammation in Alzheimer's Disease

The important role of neuroinflammation in AD has long been established (Wyss-Coray, 2006; Heneka et al., 2015). Once thought to be an innocent bystander, ramped up in the face of growing amyloid and tau pathology, inflammatory processes in the brain are now understood to contribute directly to AD pathogenesis, and to promote pathological accumulation of these protein aggregates as well (Zhang et al., 2013). Recent studies have highlighted the importance of immune-related genes and pathways in driving AD

risk and pathogenesis (Bradshaw et al., 2013; Griciuc et al., 2013; Guerreiro et al., 2013). Neuroinflammation is not only important in late-stage AD, but influences early disease progression. MCI patients with elevated inflammatory markers in the CSF are at a greater risk of conversion to AD (Tarkowski et al., 2003; Yasuno et al., 2008; Okello et al., 2009). Systemic inflammation has also been shown to influence age-related neurological functions, and to be involved in AD risk and pathological progression (Colton et al., 2006; Undén et al., 2007; Takeda et al., 2010; Kyrkanides et al., 2011.

Neuroinflammation and AD

Insults initiating in the brain are the most obvious source for inflammation driving AD risk and pathology, and have been well studied. The effects of neuroinflammation are most notably observed in changes to microglial and astrocytic activity. In the face of neuroinflammatory insults, these cells become activated and release cytokines. The activation of glial cells can be either protective or detrimental, depending on the context, and is increasingly shifted towards the pathological side of the spectrum in AD (Wyss-Coray, 2006).

Evidence suggests that inflammatory insults within the brain are associated with increased AD risk and pathological progression. Traumatic brain injury (TBI) is associated with chronic activation of microglia in the brain (Ramlackhansingh et al., 2011), and is a well-established risk factor for AD (Sivanandam and Thakur, 2012). Experimentally induced TBI has been shown to exacerbate memory deficits and elevate pathological hallmarks in AD animals (Brody and Holtzman, 2006; Tajiri et al., 2013). The loss of norepinephrine-releasing neurons, which occurs in Parkinson's disease and in diabetes (Cryer, 2013; Palma and Kaufmann, 2018), is thought to exacerbate inflammatory responses (Heneka et al., 2015). Norepinephrine possess anti-

inflammatory properties (O'Donnell et al., 2012), and its release is also reduced in normal aging and to a greater extent in AD (Zarow et al., 2003; Marien et al., 2004). In animal models, loss of norepinephrine neurons in the locus coeruleus was demonstrated to increase inflammation, promote amyloid pathology, and induce neuronal damage (Heneka et al., 2006; Kalinin et al., 2007).

Alzheimer's disease pathology in itself drives neuroinflammation. Soluble aggregates of Aβ, which are thought to be the key pathogenic species in AD (Haass and Selkoe, 2007), induce activation and pro-inflammatory cytokine release from astrocytes and microglia (White et al., 2005; Parvathy et al., 2009; Sondag et al., 2009; Hayden and Teplow, 2013). PET imaging studies indicate that higher levels of amyloid are associated with greater microglial activation, prior to onset of tau pathology (Parbo et al., 2018). Tau also contributes to inflammation in AD. Tau protein has been shown to promote microglial activation (Khandelwal et al., 2012), and genetic deletion of tau in mice was demonstrated to reduce microglial activation and neurotoxicity (Maphis et al., 2015). Based on these findings, early build-up of Aβ, owing to other risk factors and pathogenic mechanisms (including other inflammatory insults), may initiate an inflammatory feed-forward cascade of neuroinflammation within the brain, triggering increased amyloid deposition, hyperphosphorylation of tau, and neuronal damage.

ApoE genotype is also known to influence neuroinflammation (Keene et al., 2011; Tai et al., 2015). Microglia surrounding amyloid plaques have been shown to be more activated in apoE4-expressing animals than in their apoE3 counterparts (Rodriguez et al., 2014). The anti-amyloid effect of an antibody developed to target pathogenic non-lipidated apoE4 was demonstrated to require Fcγ receptor activity (Liao et al., 2018). In microglia, apoE4 is associated with elevated cytokine secretion, whereas apoE4 astrocytes secrete less proinflammatory markers than those expressing apoE3 or apoE2 (Maezawa et al.,

2006a; Vitek et al., 2009). ApoE also mediates neuroinflammation at the BBB, where activation of pericytes in mice harboring apoE4 or a genetic deletion of apoE was associated with diminished microvessels, reduced cerebral blood flow, BBB dysfunction, and neuronal damage when compared to apoE2 or E3 animals (Bell et al., 2012). Further, the authors demonstrated that apoE3, but not apoE4, from astrocytes inhibited this pericyte activation pathway through an LRP1-dependent mechanism.

Brain glia play fundamental roles in responding to and driving neuroinflammatory processes. Healthy microglia and astrocytes perform protective and supportive roles in the brain (Sofroniew et al., 2010; Kettenmann et al., 2011; Ji et al., 2013; Parkhurst et al., 2013). In one study, pharmacological depletion of microglia in an AD mouse model was associated with increased size of amyloid plaques, although no increase in the number of plaques was observed, while restoration of microglia following depletion halted the growth of these deposits (Zhao et al., 2017). However, chronic inflammatory insults can trigger aberrant phenotypes in these cells, mediating neuronal damage, an effect that is more robust in apoE4 cells (Maezawa et al., 2006b). Microglia are associated with amyloid plaques in the AD brain (Zhao et al., 2017), and express receptors that bind Aβ and promote its uptake, which triggers inflammatory cytokine release (El Khoury et al., 2003; Sheedy et al., 2013). Astrocytes can become activated early in AD pathogenesis (Kummer et al., 2014), and are also associated with amyloid plaques in animal models and human brains (Olabarria et al., 2010; Medeiros and LaFerla, 2013). Astrogliosis occurs simultaneously with atrophy of these supportive cells in AD mice (Olabarria et al., 2010), and is associated with impairment of glutamine homeostasis (Olabarria et al., 2011), which may underlie the pathogenic dysregulation of glutamate signaling in the AD brain. Microglia also respond to peripheral inflammatory signals, carried into the brain across the BBB (directly via transporters or via endothelial

cells signaling at the BBB) or by vagal nerves (Dantzer et al., 2008). The increased production of inflammatory cytokines during gliosis appears to inhibit the phagocytic capacity of microglia to eliminate Aβ from the brain, and to drive cognitive impairments (Dantzer et al., 2008; Hickman et al., 2008).

Systemic Inflammation and AD

In recent years the idea of the brain's "immune privilege" has been challenged. Communication between the peripheral immune system and the brain is well-described (Maier et al., 2001; Dantzer et al., 2008), and systemic inflammation is thought to impact BBB function and influence brain glial activity. Further, circulating immune cells have been shown to enter the brain upon systemic and neurological inflammatory insults. Systemic insults including obesity (Whitmer et al., 2008), osteoarthritis (Kyrkanides et al., 2011), diabetes (Takeda et al., 2010), and viral and bacterial infections (Mareshwari and Eslick, 2015; Licastro et al., 2016) have been shown to increase risk of AD, and to elevate pathological hallmarks of the disease. Further, MRI studies in patients with MCI and AD have found links between peripheral inflammatory markers and imaging abnormalities (Frodl and Amico, 2014). Interestingly, systemic inflammation has been shown to elicit alterations in the gut microbial composition (Vogt et al., 2017), an area of intense research in relation to AD and other neurological disorders in recent years (Ramlackhansingh et al., 2011).

ApoE has also been shown to modulate peripheral inflammation. *In vitro*, LPS elevates inflammatory markers and decreases anti-inflammatory ones to a greater extent in apoE4 macrophages than in those carrying apoE3 (Jofre-Monseny et al., 2007). In animal models and humans, apoE4 is associated with elevated inflammatory markers following LPS injection, when compared to apoE3 carriers (Gale et al., 2014).

Interestingly, apoE4 appears to protect against certain infections and their deleterious downstream effects (Wozniak et al., 2002; Azevedo et al., 2014), which may underlie the resilience of this gene in the human population, despite its detrimental effects later in life (Finch and Morgan, 2007). Importantly, Aβ has also been shown to protect against infection (Kumar et al., 2016), and two recent high-profile papers have implicated herpes virus in driving amyloid pathology as Aβ peptides appear to aggregate in an attempt to sequester viral particles and infected cells (Eimer et al., 2018; Readhead et al., 2018). Whether apoE genotype modifies the anti-microbial properties of Aβ has not yet been determined.

Cardiovascular diseases including atherosclerosis, diabetes, and hypertension, as discussed earlier in this dissertation, are well-established initiators of peripheral inflammation and macrophage activation (Pant et al., 2014; Muriach et al., 2014; Siti et al., 2015). In addition, oxidized lipids have been linked to AD pathogenesis (Testa et al., 2018), and atherosclerosis-induced inflammation has been linked to neurological disorders (Chrysohoou et al., 2018). It has been suggested that atherosclerosis and AD contribute to a feed-forward cycle in which both diseases, through vascular and inflammatory pathways, exacerbate pathology in one another (Gupta and Iadecola, 2015). Several lines of evidence support the hypothesis that vascular inflammation underlies the risk of AD associated with atherosclerosis, including the negative association of HDL levels with AD risk and the demonstrated ability of full-length apoA-I and mimetic peptides to mitigate inflammation, both in the periphery and in the brain, while simultaneously reducing neuronal damage and cognitive deficits (Paterno et al., 2004; Saczynski et al., 2007; Handattu et al., 2009; Lewis et al., 2010). Further, as discussed earlier, smoking is associated with elevated circulating inflammatory markers,

and cessation of smoking has been demonstrated to reduce inflammation, as well as risk of atherosclerosis and AD, over time (Durazzo et al., 2010; McEvoy et al., 2015).

Many immune-related genes expressed on macrophages in the periphery have been demonstrated to increase risk for AD, including TREM2, CD33, ABCA7, and TLR4 (Wang et al., 2011; Bradshaw et al., 2013; Guerreiro et al., 2013; Aikawa et al., 2018). Macrophage activity exists in a balance between beneficial and detrimental functions. These monocytes play roles in protecting against infections in the periphery and in the brain, and aid healing processes (Polfliet et al., 2001; Miron et al., 2013). However, chronic activation can cause macrophages to act aberrantly, further driving inflammatory signaling, damaging the BBB, and causing neuronal damage (Fiala et al., 2002; Ajami et al., 2011; Bogie et al., 2014). Macrophage activation is not an all-or-none phenomenon, it exists on a spectrum (Xue et al., 2014), and plays an integral role in many aspects of atherosclerotic lesion initiation and development Moore et al., 2013). Macrophages are known to infiltrate the CNS and influence amyloid deposition in animal models of AD (Simard et al., 2006). These immune cells have also been shown to degrade Aβ, an effect that is influenced by apoE genotype (Zhao et al., 2009).

TREM2 and AD

Triggering receptor expressed on myeloid cells 2 (TREM2) is a cell-surface protein that is expressed in myeloid cells, including macrophages and microglia (Klesney-Tait et al., 2006). Suspected loss-of-function mutations in TREM2 have been identified as AD risk factors in GWAS studies, increasing risk to a similar extent as apoE for carriers (Guerreiro et al., 2013; Jonsson et al., 2013; Wang et al., 2015). TREM2, as an important microglial surface receptor, has been suggested to promote microglial function and survival, and to modulate Aβ aggregation and clearance pathways (Wang et al., 2016; Xiang et al., 2016; Yuan et al., 2016; Zhong et al., 2017).

TREM2 has very rapidly become a highly studied protein in the context of AD, owing to the GWAS studies showing its association with AD risk. A series of studies sought to identify how the genetic loss of TREM2 influences AD pathology, using knock out (KO) models. KO of TREM2 in the 5XFAD mouse model of AD was associated with reduced microglial cell numbers, more apoptotic microglia, and reduced coverage of amyloid plaques by microglia (Wang et al., 2015; Wang et al., 2016). Further, these KO animals had elevated brain Aβ levels, and Aβ plaques were found to have a less compact morphology, when compared with those observed in TREM2-competent animals (Wang et al., 2015; Wang et al., 2016). These data were confirmed in another set of studies, with the complication that amyloid pathology is improved in young TREM2-KO mice, and worsened in old TREM2-KO mice (Jay et al., 2015; Jay et al., 2017). Further, the genetic depletion of TREM2 in the hTau model was associated with altered microglial morphology and increased tau pathology in 6-month old mice (Bemiller et al., 2017). In this mouse model, neurodegeneration occurs, which is an important aspect of human AD pathology that is often missing from mouse models of the disease. However, the animals used in this study were too young to study neuron loss definitively, as neuronal atrophy begins around 8 months of age in these animals (Andorfer et al., 2005). Studies using older hTau/TREM2KO mice will be needed to elucidate the role of TREM2 in influencing this phenomenon. TREM2 KO animals have also been shown to have metabolic dysfunctions, which can be corrected to improve the neuroprotective role of microglia surrounding amyloid plaques (Mazaheri et al., 2017; Ulland et al., 2017).

Efforts to identify the function of TREM2 in relation to AD pathology have been robust over the past decade. First observed in human patients in 2008, soluble TREM2 (sTREM2) is the extracellular cleavage product produced when full-length, membrane bound, TREM2 is cleaved (Piccio et al., 2008). TREM2 is known to be cleaved primarily

by ADAM10 (a disintegrin and metalloproteinase domain containing protein) (Wunderlich et al., 2013; Kleinberger et al., 2014; Thornton et al., 2017). AD risk-associated TREM2 variants including R47H and R62H occur in the extracellular immunoglobulin (Ig)-like domain of TREM2, which is part of sTREM2, while the H157Y variant occurs at the ADAM cleavage site (Guerreiro et al., 2013; Ma et al., 2014; Jiang et al., 2016; Song et al., 2017; Schlepckow et al., 2017. sTREM2 can also be produced by the alternative splicing of exon 4, which encodes the membrane-spanning and intracellular domains of the protein (Jin et al., 2014). It is important to note that following cleavage of TREM2 to release sTREM2 extracellularly, the remaining membrane-bound fragment of TREM2 is cleaved by gamma-secretase (Wunderlich et al., 2013). Since gamma-secretase is important in the cleavage of APP to produce Aβ, binding with TREM2 may influence Aβ production, although this has not yet been specifically addressed experimentally.

The relevance of TREM2 to this dissertation, beyond its role in influencing microglial function directly, is highlighted by recent reports of its binding to various lipids and lipoproteins. Polyanionic lipids have been found to interact with sTREM2, as well as apolipoprotein E (apoE), apolipoprotein J (apoJ/clusterin), apolipoprotein A-I (apoA-I), and lipoprotein particles, including HDL and LDL (Daws et al., 2003; Cannon et al., 2012; Atagi et al., 2015; Bailey et al., 2015; Park et al., 2015; Wang et al., 2015; Yeh et al., 2016; Song et al., 2017). The interaction of apoE with TREM2 does not appear to be apoE-isoform specific, although the AD risk variants R47H and R62H have reduced binding propensity with apoE and apoJ (Atagi et al., 2015; Bailey et al., 2015). The interaction between apoE and TREM2 increases microglial phagocytic activity, and TREM2 mutations are associated with reduced uptake of Aβ-lipoprotein complexes by macrophages (Yeh et al., 2016).

The use of TREM2 ectodomain-Fc fusion proteins in these studies makes it difficult to determine whether these binding interactions occur only with sTREM2, or with membrane-bound, full-length TREM2 as well, *in vivo*. However, one study led by Dr. Lars Nilsson found that while no genotype-dependant differences existed in the downstream signalling of a TREM2 reporter construct (EC_{50}; apoE2 = 27nM, apoE3 = 33nM, apoE4 = 34nM), apoE4 bound recombinant TREM2 ectodomain significantly more effectively than apoE2 (K_d; apoE2 = 13nM, apoE3 = 16nM, apoE4 = 9.5nM) (Jendressen et al., 2017). This indicates that sTREM2 and fl-TREM2 may have differential roles in regards to ligand binding, and therefore different effects on the pathological progression of AD. Further, a TREM2 ectodomain-Ig fusion protein bound lipid spots but not apoptotic or dead cell membranes, while surface expression of TREM2 was associated with binding of bacteria to cell surfaces (Daws et al., 2003; Bailey et al., 2015), indicating that the sTREM2-lipoprotein interaction may be most relevant in the extracellular space, while full-length, membrane-bound TREM2 may facilitate myeloid interaction with bacteria and other noxious agents. It was further shown that binding between apoE4 and TREM2 has a greater dependence on cooperative binding with other ligands than apoE2. Interestingly, in this study, an apoE-mimetic peptide derived of amino acids 130-149 competed with full length apoE for binding of TREM2, while a scrambled version of the peptide did not (Jendressen et al., 2017). However, the lack of a control peptide from another region of apoE makes it difficult to determine whether this peptide represents the specific binding site of the apoE-TREM2 interaction, or a more general competition between lipoproteins for TREM2 binding.

In a Swedish cohort of 25 AD patients and 25 controls from the Memory Clinic of Skåne University Hospital in Malmö, Sweden, sTREM2 levels were found to increase with age, regardless of neurological status, and positively correlated with Aβ and tau levels in

control individuals (Henjum et al., 2016). The age-related increase in sTREM2 levels has since been confirmed in another study (Suárez et al., 2016b).

Using enzyme-linked immunosorbent assay (ELISA) and mass spectrometry, several studies including participants from Sweden, the UK, Italy, and the United States have shown increased CSF sTREM2 levels in mild cognitive impairment (MCI) and AD patients when compared to cognitively normal controls (Piccio et al., 2016; Heslegrave et al., 2016; Suárez-Calvet et al., 2016b). In patients with autosomal dominant AD, sTREM2 levels are elevated beginning 5 years prior to symptom onset in patients carrying AD risk-associated TREM2 mutations, and the increase remained significant up to 5 years after onset (Suárez-Calvet et al., 2016a). Importantly, individuals harboring the AD-associated R47H TREM2 mutation had further elevated levels of CSF sTREM2 when compared to non-carriers (Piccio et al., 2016; Suárez-Calvet et al., 2016a). This differentiates AD mutations with those for frontal temporal dementia (FTD) and Nasu-Hakola disease (NHD), in which mutation carriers have reduced CSF levels of sTREM2 (Kleinberger et al., 2014; Piccio et al., 2016). The repeated finding of elevated sTREM2 levels early in disease progression as well as correlation between levels of sTREM2 and tau in the CSF has led some researchers to suggest the use of sTREM2 as a biomarker of early microglial activation in AD (Henjum et al., 2016; Suárez-Calvet et al., 2016b). The correlation between increased sTREM2 levels and onset of cognitive decline, in lieu of correlation with amyloid deposition, indicates that sTREM2 may be more functionally relevant to other aspects of AD than Aβ plaque formation, such as pathological tau progression. Indeed, recent evidence suggests that TREM2 influences tau pathology in mice (Bemiller et al., 2017).

Plasma sTREM2 levels have not been as well studied. Thus far, no difference in plasma sTREM2 levels has been observed between AD patients and controls (Kleinberger et al.,

2014), although, one study found a trend toward elevated sTREM2 protein levels in the plasma of AD patients (Hu et al., 2014). Another study found no differences in plasma sTREM2 in MS patients, despite significantly elevated CSF sTREM2 levels in the same MS patients compared with controls (Piccio et al., 2008), indicating that sTREM2 may be regulated differentially in the brain and peripheral vasculature. A significant negative correlation between monocyte TREM2 mRNA levels and cognitive performance has been described (Hu et al., 2014). Thus, elevated levels of sTREM2 in the plasma may influence cognition in AD. sTREM2 appears to interact with lipoproteins in the periphery as well as the brain, immunoprecipitating apoE, apoA-I, and apoA-II from plasma samples (Bailey et al., 2015).

The role of plasma TREM2 in human cognition has been studied to some degree. It has been demonstrated that non-obese diabetics with elevated sTREM2 have reduced cognitive performance as measured by the MMSE, whereas no such correlation was seen with sTREM2 levels in obese diabetics (Tanaka et al., 2017). Positive correlations were observed between plasma sTREM2 levels and markers of both inflammation and diabetes, indicating that peripheral sTREM2 levels are associated with increased pathology and reduced cognitive function in this cohort. These data, taken together, indicate that peripheral sTREM2 may be important in the pathological progression and treatment of neurological diseases, and further studies in this area are needed.

Summary

The collective evidence supports a critical role of systemic and neurological inflammation in the pathogenesis of AD. Cardiovascular disease-related pathways are influential in elevating inflammation and increasing risk of developing cognitive impairments. Key proteins related to this dissertation, including apoE and TREM2, interact with one

another and mediate their effects on AD pathology in part through inflammatory pathways. These inflammatory pathways and their cellular mediators are highly communicative, and disruption of the balance between pro- and anti-inflammatory mechanisms at any point can cause widespread and long-lasting dysregulation. The ability of anti-inflammatory agents to alleviate neuroinflammation and protect against AD pathology and cognitive decline is well established, and further efforts to identify effective anti-inflammatory agents may provide benefit for AD patients.

In light of the important role of immunity in AD pathology, anti-inflammatory small-molecules, as well as antibody-based therapeutics against Aβ, tau, BACE1, apoE, and other targets are in various stages of development. While animal studies of many such agents have shown marked improvement in AD pathology, clinical trials have thus far been disappointing. Nevertheless, immunotherapies make up a large portion of ongoing AD trials (Cummings et al., 2018). Other anti-inflammatory agents, including NSAIDs and statins, despite epidemiological and experimental evidence to support their use in AD, also showed no improvement in cognition in human clinical trials (Aisen et al., 2000; Simmons et al., 2002; Reines et al., 2004; Feldman et al., 2010). It is widely believed that earlier intervention and more selective inclusion criteria may be required for disease modifying therapies to reach their primary endpoints in future clinical trials designed to treat AD, as evidenced by recent anti-amyloid trials hinting at efficacy in MCI and early AD patients (http://investors.biogen.com/news-releases/news-release-details/eisai-and-biogen-announce-positive-topline-results-final; https://www.alzforum.org/therapeutics/aducanumab).

Overall Summary

The literature reviewed here highlights the diverse functions and critical importance of HDL in reducing inflammation and pathological hallmarks of AD, and in improving cognition in both animals and humans. I have also outlined the important role of both systemic and central inflammation in driving AD pathology, and explained the well-studied rationale for therapeutic benefit of small peptides designed to mimic HDL in AD. Central to this thesis is the role of apoE4 in driving AD risk and pathological progression, which has been discussed above. Chapters two and three of this dissertation will detail my work in understanding how HDL-mimetic peptides influence apoE in the context of AD, and in determining the therapeutic potential of one of these peptides, called 4F, in AD mice. The fourth and final chapter of this dissertation discusses my work in the area of neuroinflammation in AD, and in particular the potential of Minnelide, an anti-inflammatory small-molecule, in improving cognition in AD mice.

CHAPTER 2 – HDL Mimetic Peptide 4F Mitigates Aβ-Induced Inhibition of ApoE Secretion and Lipidation in Primary Astrocytes and Microglia

Introduction:

Alzheimer's disease (AD) is the leading cause of dementia in the elderly; the risk of developing AD doubles every five years after the age of 65, and over one-third of Americans over 85 are affected (Hebert et al., 2013). Although the pathogenesis of AD is not fully understood, it is widely accepted that accumulation of amyloid-β protein (Aβ) in the brain initiates a pathogenic cascade, ultimately leading to neurodegeneration and dementia (Hardy and Selkoe 2002; Mucke and Selkoe, 2012). There is currently no effective therapeutic that can halt, or even slow, the progression of AD pathology.

The apolipoprotein (apo) E ε4 allele is the strongest genetic risk factor identified to date for late-onset AD, associated with an earlier age of onset (Corder et al., 1993; Saunders et al., 1993; Strittmatter et al., 1993). In the brain, apoE is primarily secreted by astrocytes while microglia also produce apoE (Xu et al., 2006). Once secreted, apoE binds lipids and forms high-density lipoprotein (HDL)-like particles in the interstitial and cerebrospinal fluids through interactions with the ATP-binding cassette transporter A1 (ABCA1). The human apoE gene has three common alleles: ε2, ε3, and ε4 (designated apoE2, apoE3, and apoE4). The three resulting isoforms differ only at two amino acid sites in the protein. ApoE3 is the most common isoform and has a Cysteine residue at position 112 and an Arginine residue at 158. ApoE2, the rarest isoform, has Cysteine at both positions 112 and 158, whereas apoE4 has Arginine residues at both positions (Weisgraber et al., 1981).

ApoE has been extensively studied in the context of AD. It has been shown that apoE interacts with Aβ and that the level and lipidation state of apoE affect Aβ aggregation and clearance (Bu, 2009; Holtzman et al., 2012; Liao et al., 2017).

ApoE4 has been shown to be poorly lipidated compared with apoE2 and apoE3 (Kanekiyo et al., 2014; Tai et al., 2014b; Hu et al., 2015; Hanson et al., 2013; Heinsinger et al., 2016), which is thought to be the cause of many of its deleterious effects (Bu, 2009; Holtzman et al., 2012). Indeed, the receptor binding ability of apoE is dependent on its lipidation state; key residues involved in receptor binding become unburied when apoE is in a lipidated state (Chen et al., 2011). ApoE2, which reduces the risk for AD, has increased total levels of apoE compared to apoE3 or apoE4 (Cruchaga et al., 2012). This indicates that apoE level and lipidation state may be of crucial importance in combating the toxic Aβ pathway in AD pathogenesis.

Aggregated Aβ reduces the secretion of apoE and causes its cellular accumulation in astrocytes (LaDu et al., 2000; Handattu et al., 2013). The mechanism of this effect has not been fully described, although cAMP and β-adrenergic receptors have been shown to play a role (Igbavboa et al., 2006; Rossello et al., 2012). In addition, apoE binds to cell surface heparin sulfate proteoglycan (HSPG) and cell membrane associated receptors and competes with Aβ for uptake (Huang and Mahley, 2014; Fu et al., 2016). Recent studies have also demonstrated that apoE binds to the triggering receptor expressed on myeloid cells 2 (TREM2) and regulates microglial function (Atagi et al., 2015; Bailey et al., 2015; Krasemann et al., 2017; Yeh et al., 2016). It has been shown that the LDL receptor (LDLR), but not HSPG, mediates the effects of Aβ on apoE in astrocytes (LaDu et al., 2000). This highlights the direct role of the lipid metabolism pathway in AD pathogenesis. Further, the formation of apoE/Aβ complexes is observed (Wisniewski et al. 1993, LaDu et al. 1994, Tai et al. 2014b), and may increase the ability of enzymes such as insulin degrading enzyme (IDE) to degrade Aβ in the extracellular space (Russo et al., 1998). Highly lipidated apoE promotes IDE degradation of Aβ to a greater extent

than less lipidated forms (Jiang et al., 2008). Interestingly, apoE4 carriers produce less apoE/Aβ, and the complexes they do form are less stable (Tai et al., 2013). The reduced sequestration of Aβ into these complexes may hinder its degradation and lead to increased levels of toxic oligomers in the brain. However, a recent study showed that apoE minimally associates with Aβ but retains its significant influence on Aβ clearance, possibly through competing for binding to cell surface receptors (Verghese et al., 2013).

Multiple large-scale human clinical studies have found HDL levels to be highly correlated with cognitive performance late in life, and to be inversely correlated with both AD risk and severity (Hottman et al., 2014). A number of animal studies also support the role of apoA-I/HDL in AD (Lewis et al., 2010; Lefterov et al., 2010; Robert et al., 2016; Song et al., 2014). These data led to the hypothesis that apoA-I/HDL may be an attractive target for AD therapy. However, the potential of full-length apoA-I protein as a therapeutic is greatly limited by its high cost of production and lack of oral bioavailability. A series of small peptides that mimic HDL function have been developed as potential therapeutics for cardiovascular disease (Osei-Hwedieh et al., 2011; Leman et al., 2014; White et al., 2014; Getz and Reardon, 2014). The most notable of these is the peptide known as 4F, which is an 18 amino acid peptide containing 4 phenylalanine (F) residues (Ac-DWFKAFYDKVAEKFKEAF-NH2) (Segrest et al., 1983). 4F does not share sequence homology with any natural proteins but mimics the class A amphipathic helixes contained in HDL associated apolipoproteins such as apoA-I and apoE (Segrest et al., 1992). Animal studies have shown that treatment with 4F enhances HDL function and inhibits atherosclerosis (Navab et al., 2008). Intriguingly, animal experiments have also demonstrated that treatment with 4F reduces neuroinflammation and promotes cognitive function in atherosclerotic and AD mice (Buga et al., 2006; Handattu et al., 2009); the

underlying mechanisms, however, have not been elucidated. As apoE is the primary HDL-associated protein in the brain, and 4F promotes cellular lipid efflux in the periphery (Xie et al., 2010; Liu et al., 2010; Tang et al., 2006), the beneficial effects of 4F on brain function may be mediated through apoE secretion and lipidation in the brain, which has not been explored previously.

Thus, the present study aimed to determine the impact of 4F on the secretion and lipidation of apoE in primary astrocytes and microglia, and whether 4F could mitigate the effects of Aβ therein. Our results demonstrate that 4F increases the secretion and lipidation of apoE from both mouse and human astrocytes, as well as mouse microglia, and ameliorates Aβ-induced inhibition in apoE secretion and lipidation. We also show that the 4F-mediated effects depend in part on the secretory transport pathway, from the endoplasmic reticulum (ER) to the Golgi apparatus, and the function of cell surface associated receptors. Specifically, we show that 4F-mediated enhancement of apoE secretion is abolished in the absence of ABCA1 expression. These findings suggest that 4F may reduce the detrimental effects of poorly lipidated apoE in the brain and serve as a potential therapeutic agent in AD.

Methods:

Primary astrocyte and microglial culture:

This study was not pre-registered. Primary glial cells were collected and cultured as previously described (Fagan et al., 1999). Briefly, neonatal pups (total n = 84) of wild-type C57BL/6J mice (Stock No: 000664, The Jackson Laboratory, Bar Harbor, ME; RRID:IMSR_JAX:000664) were sacrificed within the first 3 days post-natal. Both sexes of the pups were used and no randomization was performed to allocate animals in the

study. The animals were anesthetized by isoflurane inhalation prior to decapitation. 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 non-essential amino acids, 2mM GlutaMAX, 2.5µg/ml amphotericin B and 50µg/ml

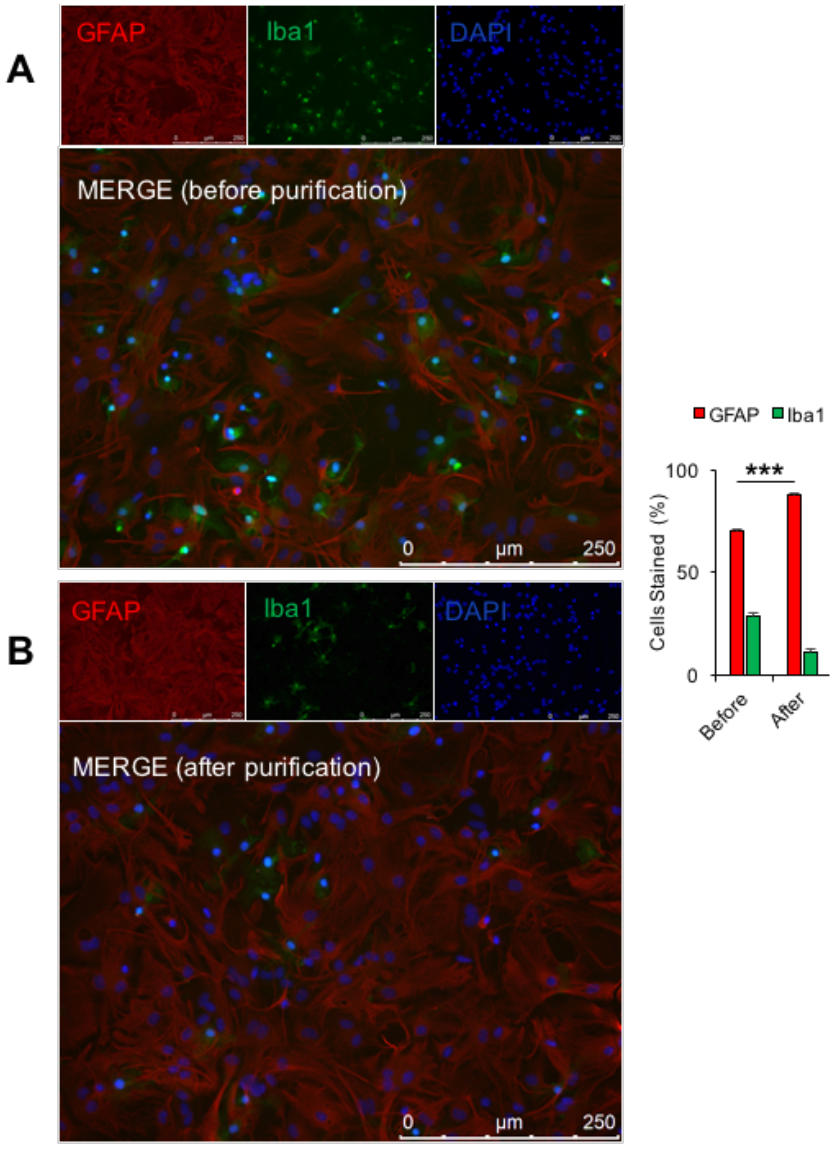


Figure 1. Purification of primary mouse astrocyte cultures. Immunofluorescence analysis of markers for astrocytes (GFAP), microglia (Iba1), and the nuclei (DAPI) in mixed glial cultures before (**A**) and after (**B**) purification (n=3 cultures). GFAP – glial fibrillary acidic protein; Iba1 - ionized calcium-binding adapter molecule 1; DAPI - 4′,6-diamidino-2-phenylindole. *** = $p < 0.001$.

gentamicin, at which point microglia were removed by shaking. Medium was replaced, and microglia were allowed to grow up an additional week, at which point they were shaken loose and removed again. Astrocytes were then passaged two times to increase purity. The purity of astrocyte and microglial cultures was determined by immunofluorescence analysis of markers specific for astrocytes and microglia (Fig. 1 and 2). Finally, cells were plated for treatment at 2x105 cells per well on poly-D-lysine (PDL) coated 12-well tissue culture plates and allowed to attach and grow for 2 days prior to experimental treatment. Human primary astrocytes were purchased from ScienCell (Carlsbad, CA; Cat# 1800) and cultured following the manufacturer's protocols. The peptides (with purity > 95%), 4F (Ac-DWFKAFYDKVAEKFKEAF-NH2), D-4F (same sequence as 4F but with D-amino acids), and Scrambled 4F (S. 4F) (Ac-DWFAKDYFKKAFVEEFAK-NH2), were purchased from American Peptide Company

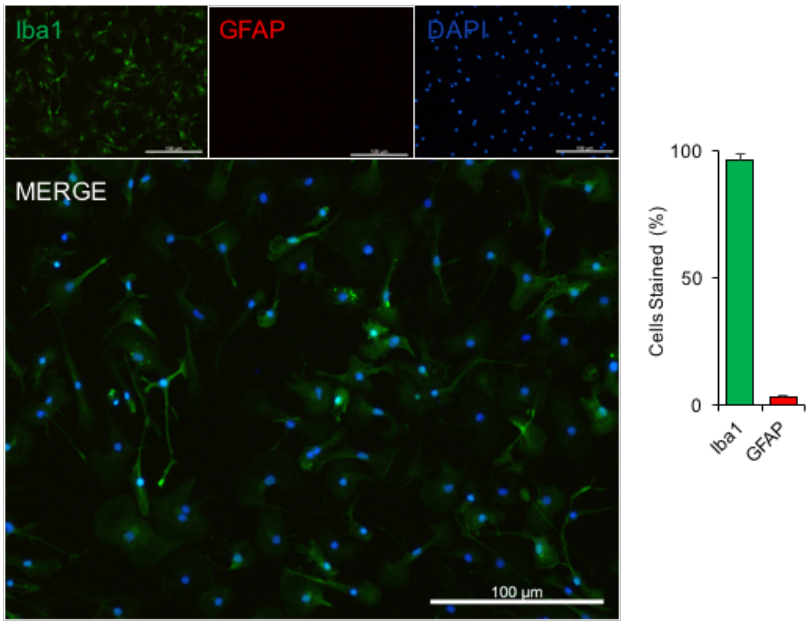


Figure 2. Purity of primary mouse microglial cultures. Immunofluorescent analysis of markers for microglia (Iba1), astrocytes (GFAP), and the nuclei (DAPI) in purified primary mouse microglial cultures (n=5 cultures). Iba1 - ionized calcium-binding adapter molecule 1; GFAP – glial fibrillary acidic protein; DAPI - 4′,6-diamidino-2-phenylindole.

(now Bachem; Sunnyvale, CA). During treatment, cells were washed twice with sterile phosphate buffered saline (PBS) and treatments were performed in serum-free OPTI-MEM supplemented with 50µg/ml gentamicin for various durations, as defined for each experiment. No blinding was performed during the experiment. All animal procedures were prospectively reviewed and approved by the Institutional Animal Care and Use Committee (IACUC protocol # 1607-33963A) of the University of Minnesota.

Gel electrophoresis and Western blot analysis:

After treatments were performed, media was collected and cells were lysed in ice-cold RIPA buffer. Media and cell lysates were then subjected to 12% SDS-PAGE. Proteins were transferred to PVDF membranes and probed with anti-mouse apoE (Santa Cruz Biotechnology, Dallas, TX; Cat# sc-6384; RRID:AB_634036) 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). ApoE from immortalized apoE3 targeted-replacement astrocytes was probed with an anti-human apoE antibody (Millipore, Burlington, MA; Cat# 178479; RRID:AB_564230). Other primary antibodies used include: anti-glial fibrillary acidic protein (GFAP) (Millipore; Burlington, MA; Cat# MAB3402; RRID:AB_94844), anti-ionized calcium-binding adapter molecule 1 (Iba1) (Wako; Richmond, VA; Cat# 019-19741; RRID:AB_839504), anti-apoJ (clusterin) (R&D; Minneapolis, MN; Cat# AF2747; RRID:AB_2083314), anti-complement C3 (MP Biomedical; Santa Ana, CA; Cat# 0855444; RRID:AB_2334469), anti-ABCA1 (Novus; Littleton, CO; Cat# NB400-105; RRID:AB_10000630), anti-LDLR (Abnova; Taipei, Taiwan, Cat# PAB8804; RRID:AB_1676510), and anti-LRP1 (Millipore Cat# MABN1796, clone 6F8; generously provided by Dr. Guojun Bu, Mayo Clinic, Jacksonville, FL).

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 125V for 5 hours. Proteins were transferred to PVDF membranes at 100V 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).

Preparation of aggregated Aβ:

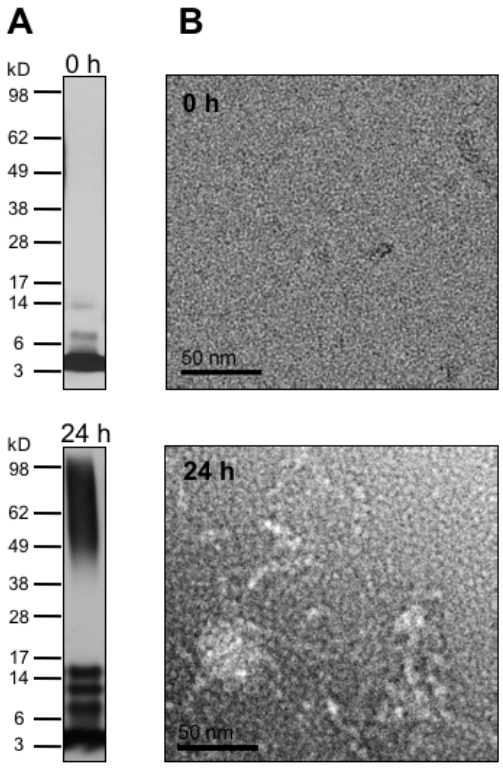


Figure 3. Characterization of Aβ preparations. Representative images of Western blot (**A**) and transmission electron microscopy (TEM) (**B**) analysis of Aβ preparations (n=2) at time 0 and after 24 h incubation.

Aggregated $A\beta_{42}$ was prepared as previously described (Stine et al., 2011). Briefly, hexafluoroisopropanol (HFIP) treated Aβ42 film, kindly provided by Dr. Mary Jo LaDu and Dr. Leon Tai (University of Illinois at Chicago), 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 at 0 and 24 hours 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 (Covance (now BioLegend); San Diego, CA; Cat# SIG-39340; RRID:AB_662806), and transmission electron microscopy (TEM) as previously described (Fitz et al., 2017) (Fig. 3). This aggregated Aβ preparation was used to treat cells.

Pharmacological manipulation:

Primary mouse astrocytes were treated with various pharmacological agents in order to understand potential mechanisms of the effects seen. 4F was prepared in sterile PBS and used at a range of concentrations from 0.1 μM to 5 μM (American Peptide Company; Sunnyvale, CA). Actinomycin D (Sigma-Aldrich; St. Louis, MO; Cat# A1410) was prepared in DMSO and used at 1 μg/ml. Cycloheximide (Sigma-Aldrich; Cat# C7698) and brefeldin A (Sigma-Aldrich; Cat# B7651) were prepared in ethanol and used at 2 μg/ml and 1μg/ml, respectively. Heparinase I (Sigma-Aldrich; Cat# H2519) and pronase (Sigma-Aldrich; Cat# P8811) were prepared in PBS and used at 5 units/ml and 10μg/ml, respectively.

Targeted deletion of ABCA1 in astrocytes using CRISPR/Cas9:

Immortalized mouse astrocytes derived from human apoE3 targeted-replacement mice (Morikawa et al. 2005), generously provided by Dr. Guojun Bu (Mayo Clinic,

Jacksonville, FL), were cultured in DMEM supplemented with 10% FBS, 2 mM GlutaMAX, 50 µg/ml gentamicin, and 10ng/ml epidermal growth factor (EGF). The cells were co-transfected with a CRISPR/Cas9 vector designed to disrupt/knock out (KO) ABCA1 gene expression, and a homology directed repair (HDR) vector, designed to incorporate genes encoding puromycin resistance as well as red fluorescence protein (RFP) into the genome in place of ABCA1. Both vectors were purchased from Santa Cruz biotechnology (Cat# sc-401086 and sc-401086-HDR, respectively). Transfected cells were visually confirmed by RFP expression, and un-transfected cells were eliminated by titration of puromycin concentration up to a final concentration of 9 µg/ml. Absence of ABCA1 protein expression was confirmed by Western blot analysis, and these cells were plated for experiments and treated with or without 4F as previously described.

Statistical Analysis:

Western blot results were quantified using Image J software. The amount of secreted apoE was analyzed as the ratio of apoE in medium to total apoE, where total apoE = apoE in medium + apoE in cell lysate, and expressed as relative percent in media with the amount in the vehicle treatment set as 100%. The total amount of apoE was normalized by tubulin when it was compared between different treatments. The amount of lipidated apoE was analyzed as the ratio of lipidated apoE to total apoE in medium, where total apoE = lipidated apoE + poorly lipidated apoE, and expressed as relative percent in lipidated form with the amount in the vehicle treatment set as 100%. Data were expressed as mean ± standard error (SE) from at least three independent experiments with each treatment in duplicate or triplicate. No sample size calculation was performed. Comparison of different treatments was performed by Student's t-test or

analysis of variance (ANOVA) (for normally distributed data), or the Mann-Whitney rank sum test (for non-normally distributed data). SigmaPlot v13.0 (Systat Software, San Jose, CA) was used for statistical analysis. $p < 0.05$ was considered statistically significant.

Results:

4F increases apoE secretion and lipidation in primary astrocytes

Due to the role of apoE in HDL-like particle formation and function, the secretion and lipidation state of apoE is highly important to its ability to perform its functions in the

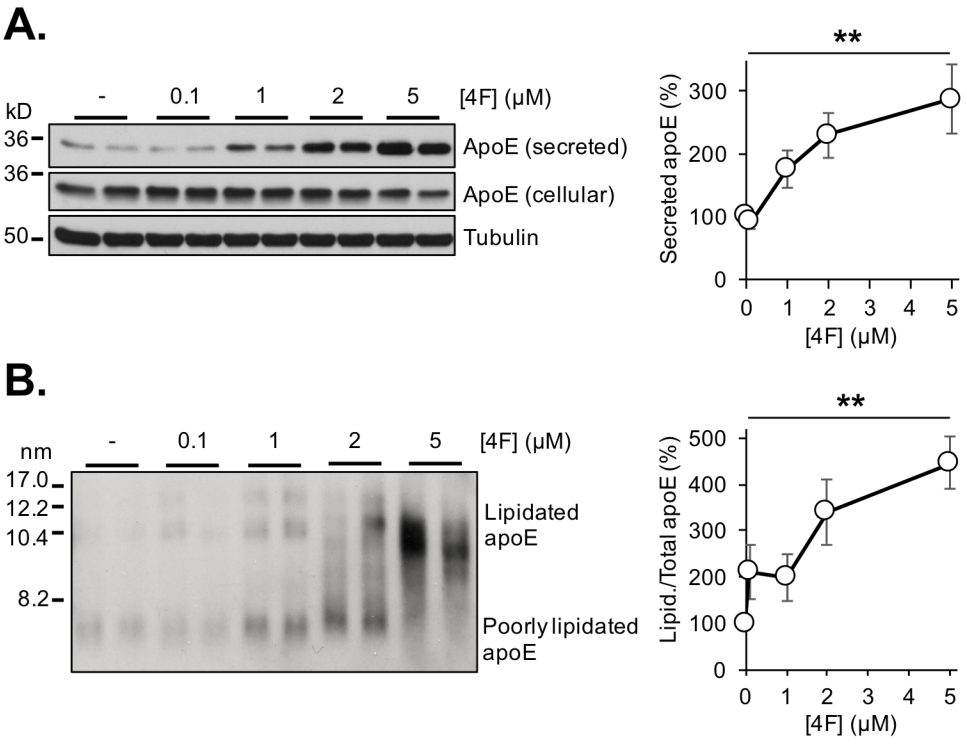


Figure 4. Dose dependent effects of 4F on apoE secretion and lipidation. Primary mouse astrocytes were treated for 24 hours with 0-5μM 4F in serum-free OPTI-MEM. (A) SDS-PAGE and (B) NDGGE were performed to determine the relative secretion and lipidation state of apoE, respectively. Tubulin was used as a loading control. Results were obtained from three independent experiments with each treatment in duplicate. ** = $p < 0.01$.

brain. ApoA-I has been shown to increase the secretion of apoE from peripheral macrophages (Rees et al., 1999) and primary mixed glia (Fan et al., 2011). We therefore hypothesized that the HDL-mimetic peptide 4F may mediate apoE secretion and

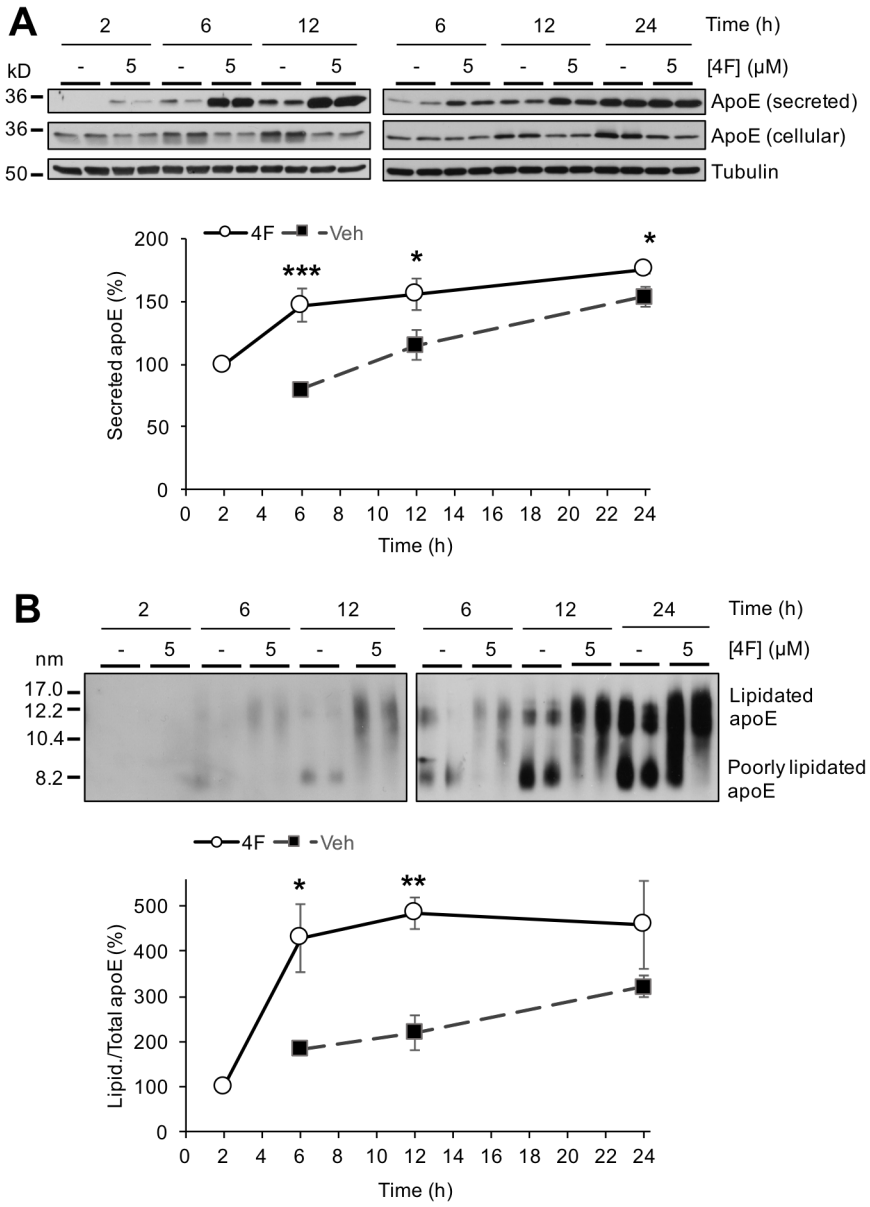


Figure 5. Time course for the effects of 4F on apoE secretion and lipidation. Primary mouse astrocytes were treated for 2-24 hours with 5μM 4F in serum-free OPTI-MEM. (A) SDS-PAGE and (B) NDGGE were performed on samples to determine apoE secretion and lipidation. Tubulin was used as a loading control. As the level of secreted/lipidated apoE at Veh-2 h was not detectable, the level at 4F-2 h was set as 100%. Results were obtained from n=2-4 independent experiments with each treatment in duplicate. * = $p < .05$, ** = $p < .01$, *** = $p < .001$.

lipidation in astrocytes. Using primary mouse astrocytes, we found that 4F induced a robust concentration- and time-dependent increase in both secretion and lipidation of apoE from these cells (Fig. 4; Fig. 5), without affecting cell survival (Fig. 6). We initially measured the levels of secreted apoE at 24 hours of treatment. We found that apoE secretion was significantly increased at 1, 2, and 5μM 4F. At 5μM 4F, the secretion of apoE was increased to approximately 300% of control (Fig. 4A). Similarly, 4F produced a dose dependent increase in apoE lipidation as well, over 400% increase at 5 μM (Fig 4B). We then used 5uM 4F to test the time-dependent effects from 2 to 24 hours, finding that both secretion and lipidation of apoE increased with time (Fig. 5). Interestingly, the 4F effect appears to begin plateauing between 6 and 12 hours of treatment (Fig. 5), although it is possible that such phenomenon was caused partly by the intrinsic saturation of the *in vitro* cell culture system or Western blotting. Despite the limitations

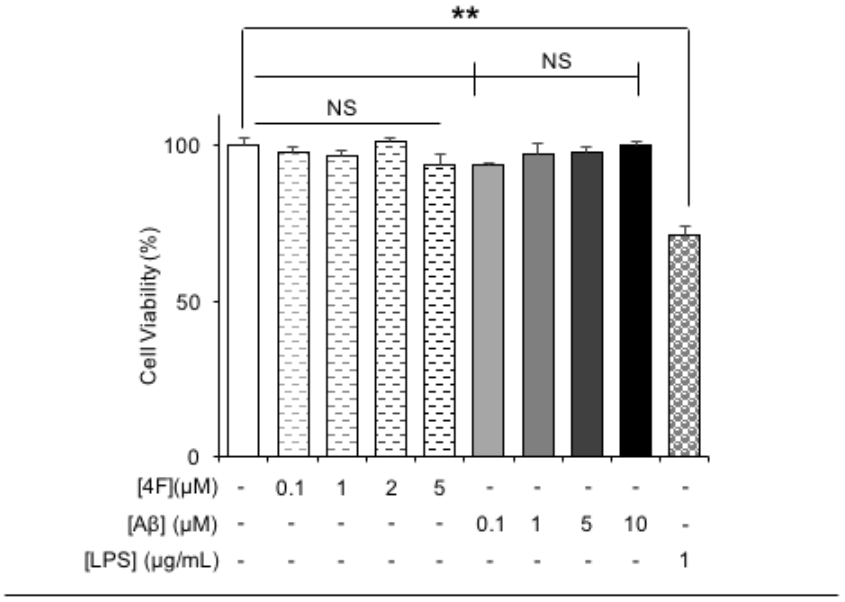


Figure 6. 4F and Aβ treatment do not affect cell survival in primary mouse astrocyte cultures. The cultures were treated with different concentrations of 4F or Aβ for 24 h and followed by the CellTiter-Blue® Cell Viability Assay (Promega; Cat# G8080) (n=3 cultures). LPS was included as control. ** = $p < 0.01$; NS, not significant.

and some variations observed, these results demonstrated that 4F treatment

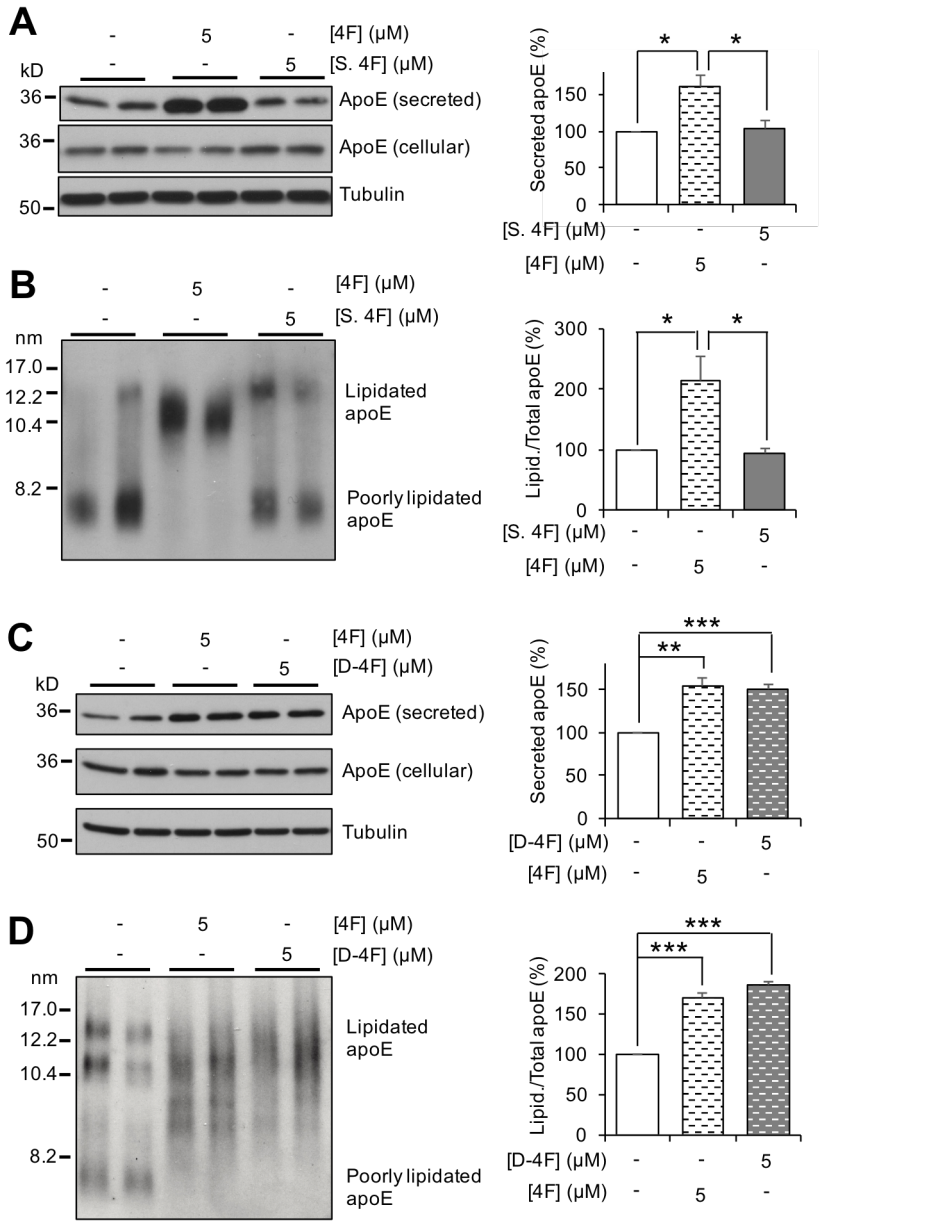


Figure 7. Scrambled 4F has no effect on apoE Secretion or Lipidation, while D-4F is equally effective as 4F. (A, B) Primary mouse astrocytes were treated for 6 hours with 5μM 4F or Scrambled 4F (S. 4F) in serum-free OPTI-MEM. (A) SDS-PAGE and (B) NDGGE were performed to determine the relative secretion and lipidation state of apoE, respectively. (C, D) Primary mouse astrocytes were treated for 6 hours with 5μM 4F or D-4F in serum-free OPTI-MEM. (C) SDS-PAGE and (D) NDGGE were performed to determine the relative secretion and lipidation state of apoE, respectively. Tubulin was used as a loading control. Results were obtained from n = 3 independent experiments with each treatment in duplicate. * = $p < .05$, ** = $p < .01$, *** = $p < .001$.

consistently increased apoE secretion and lipidation in these cells.

To test the specificity of 4F-mediated effects, we utilized a scrambled version of 4F. Scrambled 4F contains the same 18 amino acids as 4F, but in an altered sequence, which precludes the formation of the amphipathic alpha helical structure that is critical to the function of the peptide (Handattu et al., 2009). We found that scrambled 4F had no effect on apoE secretion or lipidation in comparison to 4F (Fig. 7A and 7B), confirming the specificity of 4F-mediated effects and further indicating the necessity of the particular amphipathic structure of 4F to mediate such effects. In addition, we found that D-4F, which has the same amino acid sequence as 4F but is composed entirely of the D-enantiomers of each amino acid, produced the same effect as 4F on astrocyte apoE

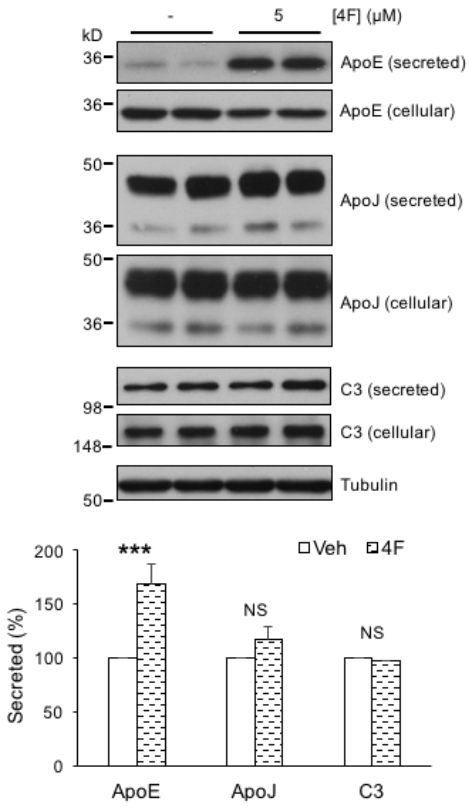


Figure 8. Specific effects of 4F on apoE secretion in primary mouse astrocytes. Western blot analysis of apoE, apoJ, and complement C3 in conditioned media and cell lysates of primary astrocytes treated with or without 4F for 24h (n=3 independent experiments with each treatment in duplicate). *** = $p < 0.001$; NS, not significant.

secretion and lipidation (Fig. 7C and 7D). These results have significant therapeutic implications because peptides D-amino acids are less susceptible to degradation of digestive/proteolytic enzymes *in vivo*.

To determine whether the effect of 4F is selective to apoE secretion, we measured the levels of two other proteins, well known to be secreted by astrocytes, apoJ (aka clusterin) and complement C3, in the medium and cell lysate. The results showed that 4F treatment did not change the secretion of these proteins (Fig. 8), indicating the selective effect of 4F on apoE secretion. Furthermore, 4F did not affect the levels of ABCA1, LDLR, or LRP1 in treated cells (Fig. 9).

Next, considering that the regulation of apoE expression differs between mouse and human cells and that mouse apoE is structurally and functionally distinct from human

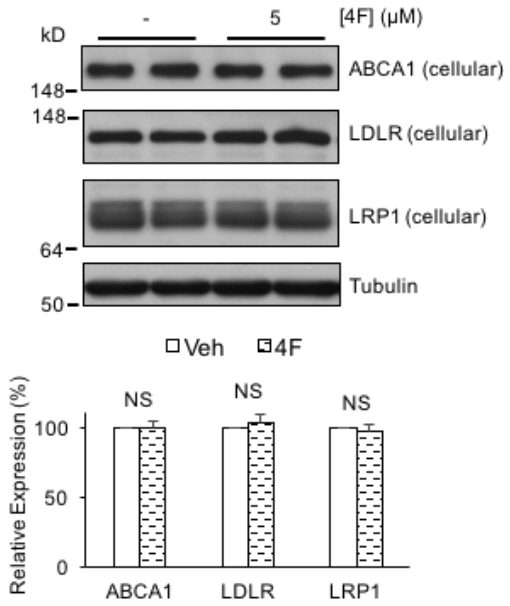


Figure 9. 4F treatment does not affect the cellular levels of ABCA1, LDLR, and LRP1 in primary mouse astrocytes. Western blot analysis of ABCA1, LDLR, and LRP1 in cell lysates of primary astrocytes treated with or without 4F for 24h (n=3 independent experiments with each treatment in duplicate). Note that the same blots as in Fig. S5 were used to probe proteins in this figure. NS, not significant.

apoE (Fagan et al., 1999; Zhu et al., 2012; Liao et al., 2015), we investigated whether the effects of 4F on apoE secretion and lipidation extend to human cells. To accomplish this, primary human astrocytes were cultured and treated with 4F for 6 and 24 hours,

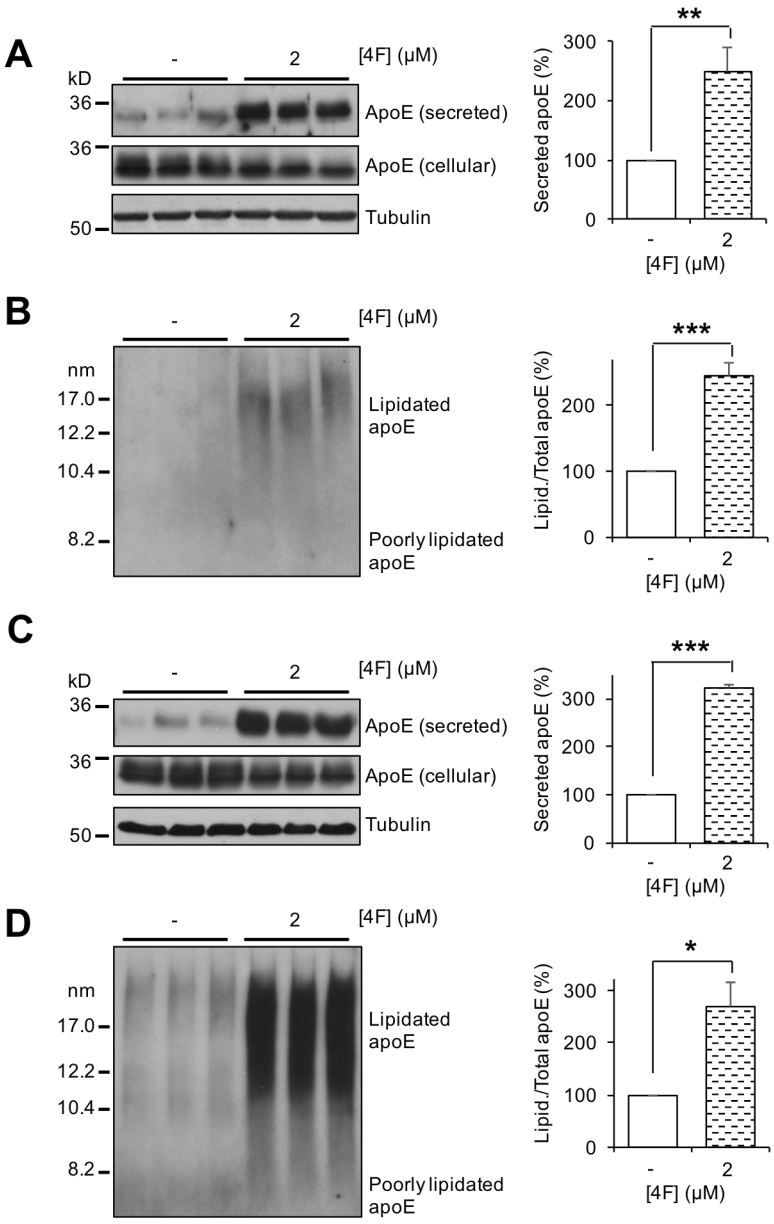


Figure 10. 4F enhances apoE secretion and lipidation in primary human astrocytes. Primary human astrocytes were treated for 6 hours (A, B) or 24 hours (C, D) with 5μM 4F in serum-free OPTI-MEM. (A, C) SDS-PAGE and (B, D) NDGGE were performed to determine the relative secretion and lipidation state of apoE, respectively. Tubulin was used as a loading control. Results were obtained from n=3 independent experiments with each treatment in triplicate. * = $p < .05$, ** = $p < .01$, *** = $p < .001$.

respectively, followed by apoE immunoblot analysis in the medium and cell lysate. The results showed that 4F treatment significantly increased the endogenous apoE secretion and lipidation in human astrocytes (Fig. 10), consistent with the findings in mouse astrocytes described above. These data demonstrate that 4F promotes apoE secretion and lipidation in both mouse and human astrocytes.

4F mitigates aggregated $A\beta_{42}$-induced inhibition of apoE secretion and lipidation

To investigate the effects of Aβ on apoE secretion and lipidation, we treated primary mouse astrocytes with different concentrations of aggregated $A\beta_{42}$, followed by immunoblot analysis. We found that aggregated $A\beta_{42}$ caused a dose-dependent decrease in apoE secretion, accompanied by accumulation of intracellular apoE in astrocytes (Fig. 11A), without affecting cell survival (Fig. 6). Furthermore, we found that apoE secreted from Aβ-treated astrocytes was less lipidated than that released by control cells (Fig. 11B). This indicates that Aβ not only suppresses the secretion of apoE, but also inhibits the lipidation of secreted apoE, thus potentially diminishing the function and stability of apoE as well. Intriguingly, co-treatment with 4F counteracts the inhibitory effects of Aβ on both apoE secretion and lipidation in primary mouse astrocytes (Fig. 5A and 11B). The Aβ-induced inhibition of apoE secretion was also observed in primary human astrocytes, and as in mouse astrocytes, co-treatment with 4F overcame the inhibitory effect of Aβ on human apoE secretion (Fig. 11C).

In addition, microglia also produce apoE, and the importance of these cells in the context of AD is highlighted by findings that apoE interacts with TREM2 and regulates microglial function (Atagi et al., 2015; Bailey et al., 2015; Krasemann et al., 2017; Yeh et al., 2016). Therefore, we tested the effects of 4F and Aβ in primary mouse microglia. Similar results

as in astrocytes were observed. 4F treatment increased, whereas Aβ treatment

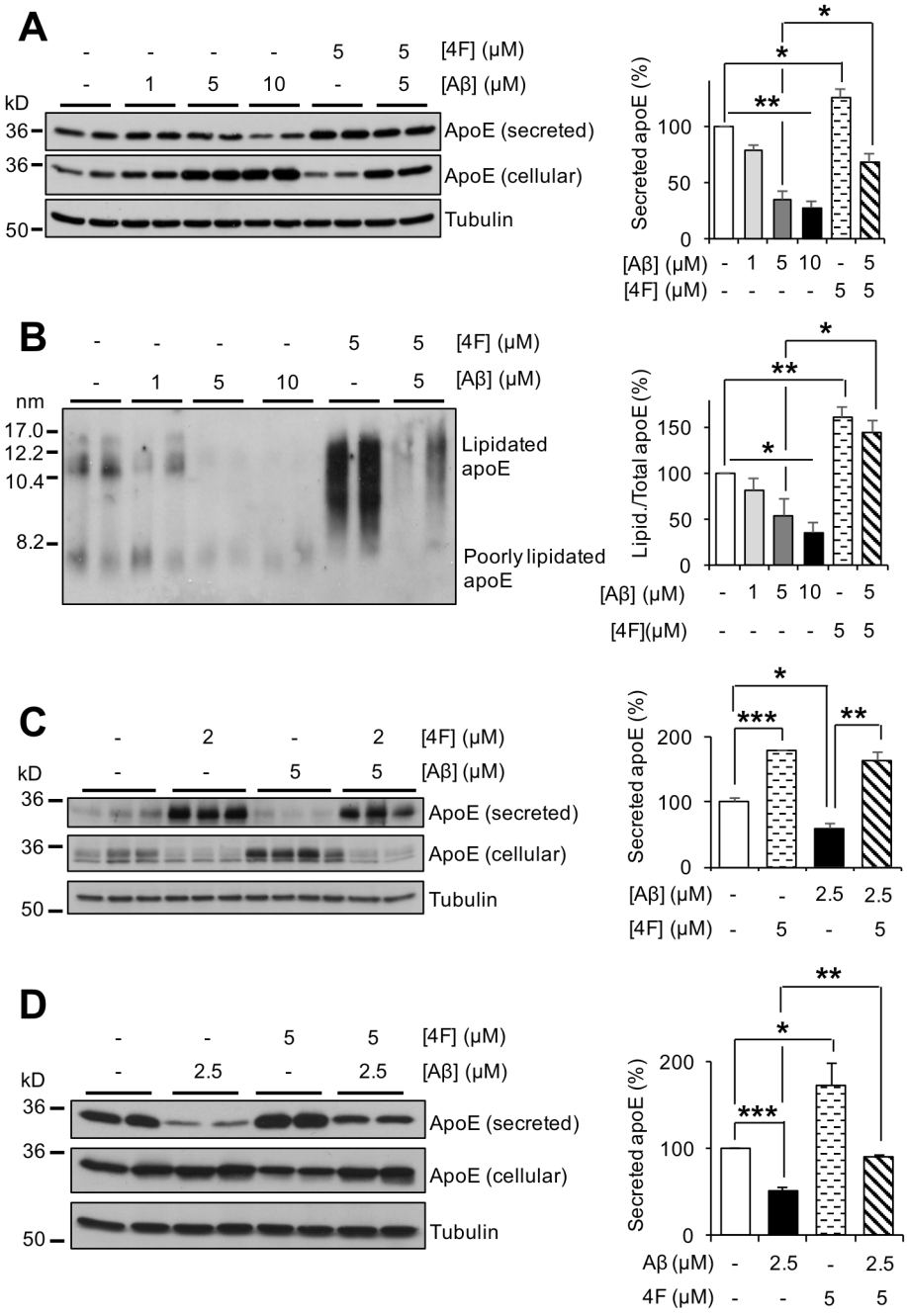


Figure 11. Aggregated Aβ inhibits apoE secretion and lipidation and 4F counteracts the inhibitory effects of Aβ in astrocytes and microglia. (A, B) Primary mouse astrocytes were cultured for 20 hours with 1-10μM $A\beta_{42}$ aggregates in absence or presence of 5μM 4F in serum-free OPTI-MEM. (C) Primary human astrocytes were treated for 24 hours with 5μM $A\beta_{42}$ aggregates and/or 2μM 4F in serum-free OPTI-MEM. (D) Primary mouse microglia were treated for 24 hours with 2.5μM $A\beta_{42}$ aggregates and/or 5μM 4F. (A, C, D) SDS-PAGE and (B) NDGGE were performed to determine the relative secretion and lipidation state of apoE, respectively. Tubulin was used as a loading control. Results were obtained from n=3 independent experiments with each treatment in duplicate or triplicate. * = $p < .05$, ** = $p < .01$, *** = $p < .001$.

decreased, apoE secretion, and co-treatment with 4F mitigated the inhibitory effect of Aβ

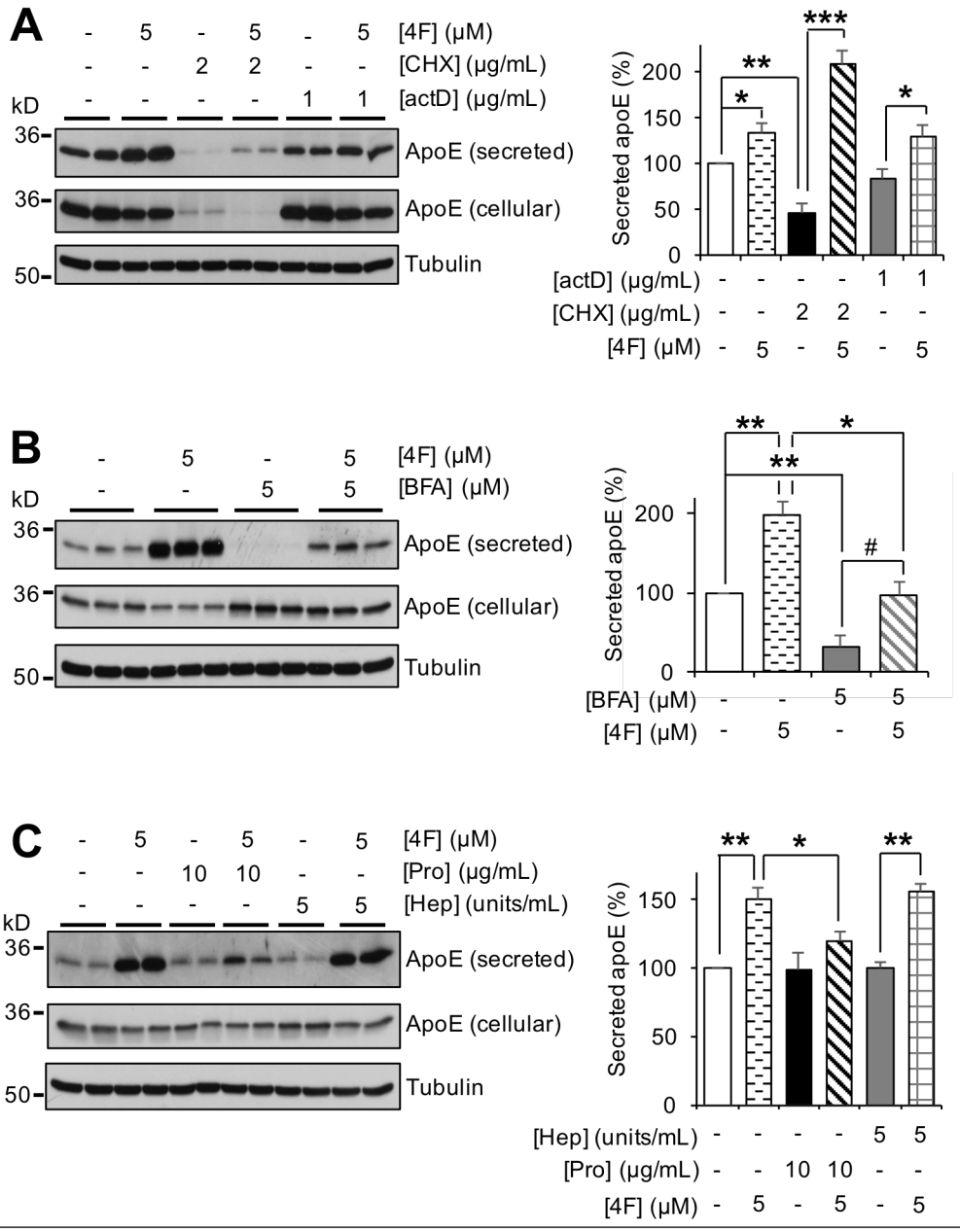


Figure 12. 4F depends on protein production, partly the canonical secretory pathway, and cell-surface receptors for its effect in primary mouse astrocytes. (A) Primary mouse astrocytes were treated for 12 hours with 1μg/mL actinomycin D (actD) or 2μg/mL cycloheximide (CHX) in serum-free OPTI-MEM with or without 4F. (B) Primary mouse astrocytes were treated for 2 hours with 5μM Brefeldin A (BFA) in serum-free OPTI-MEM in the presence or absence of 5μM 4F. (C) Primary mouse astrocytes were pre-treated for 1 hour with 5units/mL heparinase I (Hep) or 10μg/mL Pronase E (Pro) in serum-free OPTI-MEM, respectively. Cells were then washed twice with PBS and treated with 5μM 4F for 2 hours. SDS-PAGE was performed on samples to determine the relative amount of apoE secreted. Tubulin was used as a loading control. Results were obtained from n=3 independent experiments with each treatment in duplicate. * = $p < .05$, ** = $p < .01$, *** = $p < .001$; #, not significant ($p = .052$).

on apoE secretion in primary mouse microglia (Fig. 11D).

The effect of 4F on apoE secretion relies on the production of intracellular apoE but is not influenced by the process of transcription and translation per se

To determine whether new protein production is necessary for the 4F-inducted effects on apoE, we treated primary mouse astrocytes with actinomycin D (actD) and cycloheximide (CHX) to inhibit transcription and translation, respectively. ActD treatment alone did not affect the apoE protein levels (Fig. 12A), and the effects of 4F on apoE secretion were unaffected by the presence of actD, suggesting that pre-existing apoE mRNA was stable and sufficient to produce the new protein. In contrast, CHX treatment alone drastically reduced apoE levels in both the media and cell lysates (Fig. 12A). Interestingly, the residual amount of apoE produced in the presence of CHX was efficiently secreted into the medium with the treatment of 4F (Fig. 12A). Together, these results showed that the secretion of apoE promoted by 4F depended on the availability of intracellular apoE but the mechanism was not influenced by the processes of transcription and translation per se.

4F promotes apoE secretion in part through the protein transport pathway from the endoplasmic reticulum to the Golgi apparatus

To further study the mechanisms by which 4F enhances apoE secretion, we employed the use of brefeldin A (BFA), which interferes with the anterograde transport from the endoplasmic reticulum (ER) to the Golgi apparatus (Klausner et al., 1992). Treatment of primary mouse astrocytes with BFA drastically inhibited basal apoE secretion. Further, BFA treatment significantly reduced the 4F-induced effect on apoE secretion (Fig. 12B). Interestingly, in the presence of BFA, 4F treatment showed a trend increase in apoE

secretion compared to BFA alone, although this effect did not reach statistical significance (Fig. 12B). This indicates that there might be a delay in the effects of BFA on the secretory pathway during the co-treatment with 4F or there might be other pathways involved in mediating the effects of 4F on apoE secretion.

4F-stimulated apoE secretion does not involve heparin sulfate proteoglycan but requires cell surface receptors

ApoE is known to bind to heparin sulfate proteoglycan (HSPG) and other receptors on the cell surface to produce a surface-bound pool of apoE, which can be released into the media (Huang and Mahley, 2014). Therefore, we aimed to determine if 4F-stimulated apoE secretion involves the release of this extracellular matrix or receptor-bound pool of apoE. To cleave cell surface HSPG and receptors, primary mouse astrocytes were pre-treated with heparinase or pronase for 1 hour, respectively. Following a thorough wash to remove apoE released by this treatment, the cells were treated with 4F or vehicle for 2 hours in the absence of the proteolytic agents. We found that heparinase treatment had no effects on 4F-stimulated apoE secretion. 4F produced an equally significant increase in apoE secretion after heparinase treatment (Fig. 12C), indicating that 4F does not induce its effect by releasing HSPG-bound pools of apoE. Interestingly, while pronase treatment did not affect the basal level of apoE secretion, it abolished 4F-stimulated apoE secretion (Fig. 12C). Together, these results show that 4F-stimulated apoE secretion does not involve cell surface HSPG but relies on the presence of other cell surface receptors.

4F-mediated apoE secretion requires ABCA1

The inhibition of 4F-mediated apoE secretion by pronase treatment led us to question which specific cell-surface protein(s) are required for this phenomenon to occur. ABCA1 is the primary cell surface receptor responsible for the transfer of lipids onto nascent HDL particles (Oram and Heinecke, 2005). As such, we aimed to determine whether ABCA1 is required for the 4F-mediated effects on apoE to occur. We utilized immortalized mouse astrocytes with a targeted replacement of mouse apoE for human apoE3 (Morikawa et al., 2005). Using commercially available vectors based on the

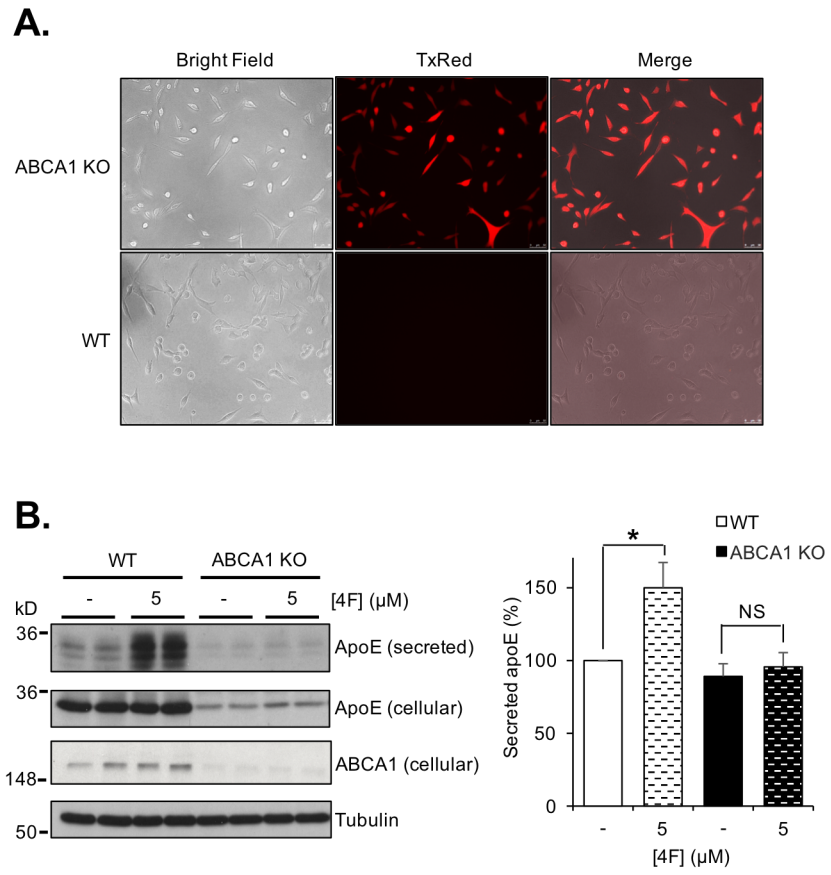


Figure 13. 4F-induced elevation of apoE secretion requires ABCA1. Immortalized apoE3 TR astrocytes were transfected with an ABCA1-specific CRISPR/Cas9 vector and a homology directed repair (HDR) vector for puromycin resistance and red fluorescence protein (RFP) to establish a stably transfected ABCA1 KO cell line. (A) Transfection is confirmed by continuous expression of RFP. Un-transfected apoE3 TR cells (WT, normal ABCA1 expression) show no RFP signal. (B) ABCA1 WT and ABCA1 KO apoE3 TR astrocytes were treated for 6 hours with or without 5μM 4F in serum-free OPTI-MEM. SDS-PAGE was performed on samples to determine the relative amount of apoE secreted, as well as ABCA1 protein expression levels. Tubulin was used as a loading control. Results were obtained from n = 3 independent experiments with each treatment in duplicate. * = $p < .05$; NS, not significant.

CRISPR/Cas9 technology (Santa Cruz Biotechnology), the ABCA1 gene was disrupted/knocked out in these cells. Transfection and loss of ABCA1 protein expression were confirmed by fluorescent imaging and Western blot analysis (Fig. 13A and 13B). We found that total levels (secreted and cellular) of apoE were markedly reduced in ABCA1 KO cells (Fig. 13B). This finding indicates that cellular production of apoE is downregulated in the absence of ABCA1, consistent with findings in animal models (Hirsch-Reinshagen et al., 2005; Koldamova et al., 2005; Wahrle et al., 2005). Compared with ABCA1-intact wild type (WT) astrocytes, 4F failed to induce apoE secretion in ABCA1 KO cells (Fig. 13B). Importantly, 4F-mediated enhancement of apoE secretion could be detected under the condition of reduced cellular apoE levels, as observed in our experiments using the translational inhibitor CHX (Fig. 12A). Thus, the results indicate that the presence of ABCA1 is required for 4F-mediated effects on apoE secretion.

Discussion:

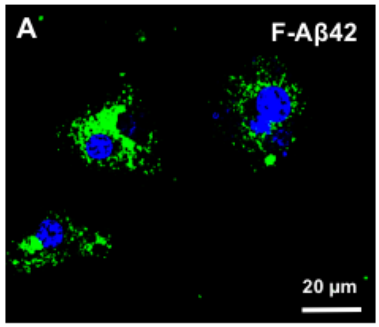
In the current study, we described the ability of 4F, an 18 amino acid HDL-mimetic peptide, to increase the secretion and lipidation of apoE in primary human astrocytes as well as primary mouse astrocytes and microglia. The primary functions of apoE require it to be part of an HDL-like particle in the brain, and mounting evidence suggests that many of its critical roles are influenced by the degree to which apoE is lipidated. ApoE4, the primary genetic risk factor for late onset AD, has been shown to be poorly lipidated compared with apoE2 and apoE3 (Kanekiyo et al., 2014; Tai et al., 2014b; Hu et al., 2015; Hanson et al., 2013; Heinsinger et al., 2016). As the lipidation status of apoE affects its function and receptor binding capacity (Bu, 2009; Koldamova et al., 2014),

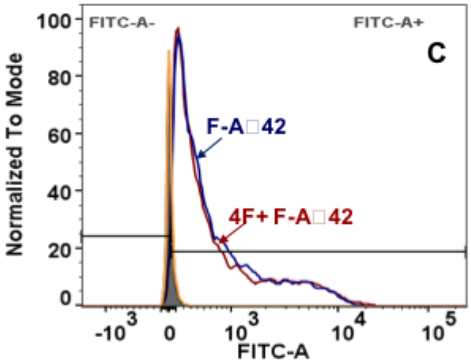
deficits in lipidation may underlie the pathogenic effects of apoE4. Therefore, increasing the lipidation of apoE may be a viable therapeutic avenue for AD treatment.

A number of studies have shown that oligomeric species of Aβ correlate better with cognitive impairment than Aβ plaque burden in AD (Haass and Selkoe, 2007; Lesne et al., 2013; Selkoe and Hardy, 2016). Aβ has also been previously shown to inhibit apoE secretion from astrocytes (LaDu et al., 2000; Igbavboa et al., 2006; Handattu et al., 2013). Our results corroborate these previous findings on the inhibition of apoE secretion by Aβ in both mouse and human astrocytes as well as in mouse microglia, and further demonstrate that lipidation of secreted apoE from primary mouse astrocytes is also inhibited by Aβ, which may reduce apoE function, stability, and receptor binding capacity. The interaction between Aβ and apoE may be a crucial part of early pathogenesis, in which early Aβ insults reduce apoE secretion and lipidation, leading to reduced Aβ clearance and thus elevated levels of aggregated Aβ in the brain. This in turn may create a vicious feed-forward cycle in which these elevated levels of aggregated Aβ cause a further decline in apoE secretion and lipidation. Blocking this circular pathway with a pharmacological agent that increases apoE secretion and lipidation may slow or even halt the progression of AD. Interestingly, Verghese and colleagues showed that the association between apoE and Aβ was minimal in the cerebrospinal fluids in human apoE targeted-replacement mice and that apoE-deficient astrocytes cleared Aβ from the medium more efficiently than apoE-expressing astrocytes (Verghese et al., 2013). They further showed that apoE and Aβ compete for the same LRP1-mediated clearance pathway, suggesting that reducing apoE levels would enhance Aβ clearance (Verghese et al., 2013). We examined the effect of co-treatment of 4F on Aβ uptake in primary mouse astrocytes by confocal microscopy and flow

cytometry as described previously (Omtri et al., 2012). The results showed that 4F did not interfere with Aβ uptake (Fig. 14). Importantly, chronic treatment with 4F resulted in a significant reduction of amyloid deposition in the APP/PS1 model of AD (Handattu et al., 2009), indicating that 4F may facilitate brain Aβ clearance through multiple mechanisms *in vivo*.

When the effect of Aβ on reducing secretion of apoE was described initially, it was postulated that the Aβ-induced effect might be protective, based on the assumption that an increase in the cellular production of apoE counteracts the detrimental effects of Aβ (LaDu et al., 2000; Igbavboa et al., 2006). However, more recent studies indicate that apoE must be secreted and properly lipidated in order to perform its canonical functions, including lipid/cholesterol transport, synapse regeneration, immune modulation, and





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Treatments	MFI ± SD
Control	84.8 ± 6.8
4F alone	81.8 ± 5.2
F-A□42	431.3 ± 51.7
4F + F-A□42	423.7 ± 35.2

Figure 14. 4F treatment does not affect the uptake of F-Aβ42 in primary mouse astrocytes. Confocal microscopy (**A, B**) images and histograms from flowcytometry (**C**) of primary astrocytes treated with 1.6μM F-Aβ42 alone (**A**) or in combination with 5μM 4F (**B**) for 1 h at 37 °C . Blue: DAPI, Green: F-Aβ42. (D) Flowcytometry data analysis: FITC positive and negative cells were initially gated and then median fluorescence intensity (MFI) of FITC positive cells were obtained. Data is presented as average ± SD (n= 3 cultures).

clearance/degradation of Aβ (Kanekiyo et al., 2014; Tai et al., 2014b; Hu et al., 2015; Hanson et al., 2013). As such, Aβ-induced accumulation of intracellular apoE and simultaneous inhibition of secretion is considered detrimental. We have found that co-treatment of astrocytes with 4F mitigates the Aβ-induced inhibitory effects on apoE secretion and lipidation. In the presence of 4F, astrocytes exposed to Aβ secrete similar amounts of apoE as control cells, and the secreted apoE is lipidated as efficiently as in the controls. Consistent with our findings, it has been shown that a hybrid apoE/apoA-I-mimetic peptide, Ac-hE18A-NH2, mitigated the inhibitory effect of Aβ in the U251 astrocyte cell line (Handattu et al., 2013). These results suggest that 4F or related apo mimetic peptides can counteract the detrimental effects of Aβ on astrocytes, potentially serving as a protective agent against AD. These findings *in vitro* have significant implications *in vivo*. Although it remains a topic of debate whether an increase or decrease in total apoE levels is protective against AD, improving the lipidation state of apoE ameliorates cognitive deficits in the presence or absence of amyloid pathology regardless of apoE genotype (Cramer et al., 2012; Fitz et al., 2013; Boehm-Cagan and Michaelson, 2014).

In the present study, we showed that 4F treatment consistently produced a robust increase in apoE secretion and lipidation in astrocytes throughout the experiments. The mechanisms by which 4F promotes apoE secretion were explored with the use of pharmacological agents and CRISPR/Cas9-mediated gene editing approaches. The effect of 4F occurs in part through the classical ER-Golgi secretion pathway, as inhibition of this pathway by BFA partially blocks the 4F-induced enhancement of apoE secretion. Our experiments with heparinase demonstrate that cell surface HSPG-associated apoE does not contribute to the effect of 4F on apoE secretion, which is in line with a previous

study showing that the Aβ-induced elevation of intracellular apoE does not depend on interactions with HSPG (LaDu et al., 2000). However, removal of other cell surface receptors with pronase abolishes the increase of apoE secretion by 4F in primary astrocytes. Furthermore, using ABCA1 KO astrocytes generated by using CRISPR/Cas9 technology, we show that the effect of 4F on apoE secretion requires the presence of ABCA1. Notably, it was reported recently that an apoE-mimetic peptide ameliorated apoE4-driven cognitive and brain pathologies through activation of ABCA1 (Boehm-Cagan et al., 2016). These findings corroborate the importance of ABCA1 in apoE-targeted therapeutic development.

Highlighting the importance of ABCA1 in the context of AD, several studies have shown that ABCA1 deficiency exacerbates amyloid pathology in AD mice (Hirsch-Reinshagen et al. 2005; Koldamova et al., 2005; Wahrle et al., 2005). ABCA1 KO mice exhibit drastically reduced apoE levels and lipidation. In contrast, apoE KO AD mice have reduced amyloid deposition (Bales et al., 1997; Irizarry et al., 2000) and tau pathology (Shi et al., 2017). Furthermore, immunotherapy against apoE was associated with reduced Aβ deposition in AD mice (Kim et al., 2012), an effect that has recently been recapitulated using an antibody targeting only non-lipidated forms of apoE (Liao et al. 2018). These findings, together, indicate that the lipidation state of apoE may influence AD pathology more so than the absolute level of apoE.

In line with these findings, ABCA1 overexpression is associated with elevated apoE levels and lipidation, and attenuates AD pathology (Wahrle et al., 2008). Bexarotene and other nuclear receptor agonists, which upregulate genes including ABCA1, have been shown to increase apoE level and lipidation, while reducing amyloid pathology in mice (Cramer et al., 2012, Zelcer et al., 2007; Skerrett et al., 2014; Donkin et al., 2010,

Corona et al., 2016), although the effect on amyloid pathology was not observed in all studies (Veeraraghavalu et al., 2013; Tesseur et al., 2013). Importantly, these agents produce deleterious peripheral effects on triglyceride production and liver health (Hong and Tontonoz, 2014; Tousi, 2015; Tai et al., 2014a), as well as off-target effects inherent when targeting promiscuous transcription factors, indicating that alternative methods of increasing apoE lipidation may provide safer and greater benefits to those suffering from AD.

The potential for development of full-length HDL-associated apolipoproteins such as apoA-I or apoE as therapeutics is limited by their size, structure and post-translational modifications, making small HDL-mimetic peptides, such as 4F, attractive candidates in this regard. Notably, unlike other HDL-mimetic peptides (Leman et al., 2014; White et al., 2014), 4F has been tested in three human clinical trials with 50, 152 and 62 individuals, respectively, for cardiovascular disease; 4F was found to be safe and well-tolerated when administered orally or by injections, and improved HDL anti-inflammatory properties (Bloedon et al., 2008; Watson et al., 2011; Dunbar et al., 2017). Further, our finding that D-4F improves apoE secretion and lipidation to the same extent as 4F has significant implications for therapeutic development, because D-4F is orally bioavailable with a longer half-life than L-4F *in vivo* (Navab et al., 2005).

Our studies indicate that the small peptide, 4F, may harbor the beneficial effects of apoA-I/HDL in a form that is both more economically viable and much easier to deliver to the brain. Notably, D-4F, in the presence of a low/ineffective dose of a statin drug, has been shown to mitigate memory deficits and amyloid pathology in the APP/PS1 model of AD (Handattu et al., 2009). In that study, D-4F with pravastatin was administered orally in drinking water to 4- to 5-month old male APP/PS1 mice for 3 months, using scrambled

D-4F with pravastatin and drinking water alone as controls, followed by behavioral assessment and biochemical analyses. The results showed that treatment with D-4F+pravastatin improved the learning and memory performance of APP/PS1 mice in the Morris water maze test and led to a >50% reduction in Aβ load compared with the controls (Handattu et al., 2009). Further analysis showed that D-4F+pravastatin treatment did not alter APP expression or proteolytic processing but was associated with a decrease in glial activation and inflammatory markers in the brain. Thus, it was concluded that D-4F+pravastatin treatment inhibits Aβ deposition and improves cognitive function through exerting anti-inflammatory actions in the brain (Handattu et al., 2009). These findings strongly support the beneficial effects of 4F treatment *in vivo*, although a study with 4F in the absence of any statin drug will be needed for confirmation. In addition, the role of apoE was not investigated in that study. As apoE plays a pivotal role in Aβ metabolism and immune modulation, and based on the findings in the present study, we hypothesize that the enhancement of apoE secretion and lipidation may underlie the beneficial effects of 4F treatment in AD mice. Further studies will be required to test this hypothesis.

Targeting apoE is a promising approach for AD therapy. However, simply altering the level of apoE may not be sufficient, particularly in individuals carrying the apoE4 allele. Mounting evidence shows that the lipidation status of apoE dictates its function and relative contribution to AD risk. The present study has demonstrated that the HDL-mimetic peptide, 4F, promotes secretion and lipidation of apoE from astrocytes and microglia, in both the presence and absence of Aβ, suggesting that HDL-mimetic peptides such as 4F may serve as effective apoE-modulating agents against AD.

CHAPTER 3 – HDL Mimetic Peptide 4F Mitigates ApoE4-Associated Lipidation and Memory Deficits

Introduction

The apolipoprotein (apo) E ε4 allele is well-established as the strongest genetic risk factor for sporadic Alzheimer's disease (AD). ApoE4 carriers experience an earlier age of AD onset (Corder et al., 1993; Saunders et al., 1993; Strittmatter et al., 1993). ApoE is produced peripherally, and in the brain, where it is primarily secreted by astrocytes (Xu et al., 2006). Secreted apoE interacts with lipids and the ATP-binding cassette transporter A1 (ABCA1) to form high-density lipoprotein (HDL)-like particles in the interstitial and cerebrospinal fluids. In humans, three common variants of apoE exist: ε2, ε3, and ε4 (designated apoE2, apoE3, and apoE4). These isoforms differ at two amino acid sites in the protein. ApoE3, the most common isoform, has a Cysteine residue at position 112 and an Arginine residue at 158, whereas apoE2, which is protective in AD, has Cysteine at both positions, and conversely, apoE4 has Arginine residues at both positions (Weisgraber et al., 1981).

ApoE is known to interact with Aβ and to influence its aggregation and clearance, a function that depends upon the level and lipidation state of apoE (Bu 2009, Holtzman et al. 2012; Liao et al., 2017), although the degree to which apoE directly interacts with Aβ has been called into question (Verghese et al., 2013). Structural deficits have been identified in apoE4, compared to apoE3, resulting in inefficient lipidation and reduced stability (Hatters et al., 2006). ApoE4 protein binds its receptors with lower affinity, promotes Aβ aggregation and deposition, and inhibits clearance of Aβ, when compared to apoE2 and apoE3 (Kanekiyo et al., 2014; Tai et al., 2014b). Recently, it was shown that apoE4 also plays a critical role in the development of tau pathology in AD (Shi et al.,

2017). Other pathways thought to influence apoE4-mediated elevation of AD pathogenesis include cholesterol/lipid metabolism, synaptic plasticity, cell signaling, and inflammation. It is likely that apoE4-mediated deleterious effects owe not only to loss of neuroprotection, but also arise due to toxic gain of function (Huang and Mucke, 2012).

Studies show that apoE4 is more poorly lipidated than apoE2 and apoE3 (Kanekiyo et al., 2014; Tai et al., 2014b; Hu et al., 2015; Hanson et al., 2013; Heinsinger et al., 2016), which is thought to underlie many of its detrimental effects (Bu, 2009; Holtzman et al., 2012). Further, the lipidation status of apoE influences tertiary structure and thereby its receptor binding capacity (Chen et al., 2011). Interestingly, the selective targeting of non-lipidated apoE3 and apoE4 with passive immunization was demonstrated to reduce amyloid pathology in a mouse model of AD (Liao et al., 2018).

As discussed in Chapter 2 of this dissertation, our previous studies in primary mouse astrocytes showed that a small (18 amino acid) peptide designed to mimic the beneficial anti-inflammatory functions of high density lipoprotein (HDL), called 4F, increases the lipidation and secretion of apoE and mitigates Aβ-induced deficits therein (Chernick et al., 2018). Importantly, our studies using human primary astrocytes confirmed the relevance of these effects to human apoE, which differs from mouse apoE in structure and function.

This study was undertaken to determine the influence of 4F on secretion and lipidation of the three common human apoE variants, as well as the potential of the mimetic peptide to ameliorate both apoE4-mediated and AD-related cognitive deficits and pathology. *In vitro* studies confirm existing knowledge on the genotype-specific lipidation status of human apoE (apoE2 > apoE3 > apoE4), and show that 4F rescues apoE4 lipidation,

restoring it to the same level as that of apoE2. Further, we describe the novel finding that apoE4 is more susceptible to Aβ-induced inhibition of apoE lipidation, when compared to apoE2, and that 4F counteracts this effect. We found that in the SwDI model of CAA, acute daily i.p. injection of 4F for 1 week reduces soluble Aβ levels, while chronic 12-week treatment improves apoE lipidation in the brain, and in male animals, improves spatial memory retention. Finally, daily i.p. injection of D-4F for three weeks mitigated apoE4-induced cognitive deficits in apoE targeted replacement mice. Given the aging population and recent high-profile failures of promising agents to halt or slow AD progression in clinical trials, redoubled efforts to identify potential therapeutics for this devastating disease are needed. Targeting of apoE, and in particular its lipidation status, is an enticing prospect that has gained significant attention in recent years.

Methods

Animals and treatments

SwDI mice expressing Swedish, Dutch, and Iowa mutations in the amyloid precursor protein (APP) (C57BL/6-Tg(Thy1-APPSwDutIowa)BWevn/Mmjax; MMRRC Stock No: 34843-JAX) (Davis et al., 2004), were obtained from Jackson laboratory in Bar Harbor, ME.

In this study, SwDI mice (male and female; n = 14) were treated once daily with sterile PBS or D-4F by intraperitoneal (i.p.) injection for 12 weeks. The dose was 5mg/kg body weight for the first 5 weeks, and was lowered to 2.5mg/kg for the remaining 7 weeks. D-4F was resuspended in sterile PBS to a concentration of 2.5mg/mL (1.25mg/mL after week 5), such that for an average 50g mouse, the injection volume would be 100uL/day. Mice were weighed once weekly, starting with the day prior to the 1st injection, and the

volume administered was adjusted accordingly for the following week. PBS-treated animals received an equivalent injection volume, determined by the same means. A second cohort of SwDI animals (male and female; n = 8) was treated similarly to above, with PBS (n = 4) or D-4F (n = 4) for 1 week at an i.p. dose of 2.5mg/kg.

Homozygous human apoE2, apoE3, and apoE4 targeted-replacement mice were purchased from Taconic for experimental treatment, followed by behavioral assessments. Primary glial cultures were established from neonatal (1 to 3-day-old) pups for *in vitro* studies.

In this study, apoE2, apoE3, and apoE4 animals (male and female; n = 37) were treated once daily with sterile PBS or D-4F by intraperitoneal (i.p.) injection, at a dose of 2.5mg/kg body weight, for 3 weeks. D-4F was resuspended in sterile PBS to a concentration of 1.25mg/mL, such that for an average 50g mouse, the injection volume would be 100uL/day. Mice were weighed once weekly, starting with the day prior to the 1st injection, and the volume administered was adjusted accordingly for the following week. PBS-treated animals received an equivalent injection volume, determined by the same means.

Strict adherence to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health was employed during the design and performance of this study. All animal procedures were prospectively reviewed and approved by the Institutional Animal Care and Use Committee of the University of Minnesota (Protocol number: 1607-33963A). All efforts were made to minimize animal suffering throughout the study.

Immortalized astrocyte culture

Immortalized mouse astrocytes derived from human apoE3 targeted-replacement mice (Morikawa et al., 2005), generously provided by Dr. Guojun Bu (Mayo Clinic, Jacksonville, FL), as well as cells from the same origin expressing apoE2 and apoE4, generously provided by Dr. William Rebeck (Georgetown University, Washington, D.C.) were cultured in DMEM supplemented with 10% FBS, 2mM GlutaMAX, 50µg/ml gentamicin, 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 50µg/ml gentamicin for various durations.

Primary astrocyte culture

Primary glial cells were collected and cultured as previously described (Fagan et al. 1999). Briefly, neonatal pups of human apoE2, apoE3, and apoE4 targeted-replacement mice were sacrificed within the first 3 days post-natal. Brains were removed from the skull, after which cortical and hippocampal glia were dissociated into a single-cell suspension and cultured for two weeks in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 16mM HEPES buffer, 0.1mM non-essential amino acids, 2mM GlutaMAX, and 50µg/ml gentamicin, at which point microglia were removed by shaking. Medium was replaced, and microglia were allowed to grow up for one more week, at which point they were shaken loose from the astrocyte monolayer and removed again. Astrocytes were then passaged two times to increase purity. Lastly, cells were plated at $2x10^5$ cells per well on PDL coated 12-well tissue culture plates, allowed to attach and grow for 2 days, and then exposed to experimental treatments. To

treat the cells, they were washed twice with sterile phosphate buffered saline (PBS) before adding experimental agents in serum-free OPTI-MEM supplemented with 50µg/ml gentamicin for various durations. All animal procedures were prospectively reviewed and approved by the Institutional Animal Care and Use Committee (IACUC protocol # 1607-33963A) of the University of Minnesota.

Gel electrophoresis and Western blot analysis

After treatments were performed, media was collected and cells were lysed in ice-cold RIPA buffer. Media and cell lysates were then subjected to 12% SDS-PAGE. Proteins were transferred to PVDF membranes and probed with anti-human apoE (Calbiochem; San Diego, CA; Cat# 178479) and tubulin (Sigma-Aldrich, St. Louis, MO; Cat# T5168) antibodies, followed by HRP-conjugated secondary antibody and chemiluminescence detection using ECL reagents (GE Healthcare, Little Chalfont, U.K.).

Non-denaturing gradient gel electrophoresis (NDGGE) was used to assess apoE lipidation. Fresh media was run on 4-20% polyacrylamide tris-glycine gels (Invitrogen, Carlsbad, CA) in the absence of SDS, reducing agents or sample boiling at 125V for 5 hours. Proteins were transferred to PVDF membranes at 100V 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).

Blue NativePAGE was used to determine the lipidation of apoE in brain homogenates, as previously described (Hu et al., 2015). Briefly, flash-frozen cortical tissue was thawed on ice and homogenized in ice-cold PBS, with protease and phosphatase inhibitors

present, using 15 strokes through a 25-gauge needle. Homogenates were clarified by spinning at 10,000 x g for 10 minutes, and then samples were run on 4-16% NativePAGE Bis-Tris gels (ThermoFisher; Cat. # BN1004BOX) following the manufacturer's instructions. Proteins were transferred to PVDF membranes in tris-glycine buffer with 10% ethanol at 100V for 1.5 hours. Ponceau staining was used to visualize the high molecular weight marker (Amersham), and blots were then probed for apoE using the same antibody as previously described, followed by chemiluminescent detection.

Preparation of 4F and X-4F

The peptides (with purity > 95%), 4F (Ac-DWFKAFYDKVAEKFKEAF-NH2) and X-4F (a modified version of the 4F peptide, designed to enhance activity) were purchased from and synthesized by the American Peptide Company (now Bachem; Sunnyvale, CA), and resuspended from powder in sterile PBS. The peptides were used at a range of concentrations, from 0.1μM to 5μM, depending upon the experimental design.

Preparation and characterization of aggregated Aβ

$A\beta_{42}$ was aggregated as previously described (Stine et al., 2011). Briefly, hexafluoroisopropanol (HFIP) treated $A\beta_{42}$ film, kindly provided by Dr. Mary Jo LaDu and Dr. Leon Tai (University of Illinois at Chicago), was resuspended in fresh dry DMSO, diluted in phenol-free F12 cell culture media to a concentration of 100μM, and incubated at 37°C for 24 hours.

Aliquots of the Aβ preparation were subjected to LDS-NuPAGE (4-12% bis-tris gels; ThermoFisher; Waltham, MA; Cat# NP0322BOX) and then examined by immunoblot analysis with the anti-Aβ antibody, 6E10 (Covance (now BioLegend); San Diego, CA;

Cat# SIG-39340; RRID:AB_662806). The aliquots were also studied by transmission electron microscopy (TEM), as previously described (Fitz et al. 2017). This aggregated Aβ preparation was used to treat cells.

Behavioral analysis

Three behavioral functions related to AD were assessed (spatial learning and memory, exploration of environmental stimuli, and anxiety), and the testing procedures employed herein have previously been described in detail (Lewis et al., 2010; Cheng et al., 2013). The testing was performed sequentially, starting with three days of the open field test for locomotor activity, immediately followed by two days of the elevated plus-maze test for anxiety levels, and lastly, after two days without external stimulation/activity, the mice were tested for spatial learning and memory in the Morris water maze (days 8–13). All equipment and software used in these assessments was purchased from SD Instruments (San Diego, CA).

Statistical Analysis

Western blot results were quantified using Image J software. The amount of secreted apoE was analyzed as the ratio of apoE in medium to total apoE, where total apoE = apoE in medium + apoE in cell lysate, and expressed as relative percent in media with the amount in the vehicle treatment set as 100%. The total amount of apoE was normalized by tubulin when it was compared between different treatments. The amount of lipidated apoE was analyzed as the ratio of lipidated apoE to total apoE in medium, where total apoE = lipidated apoE + poorly lipidated apoE, and expressed as relative percent in lipidated form with the amount in the vehicle treatment set as 100%. Data were expressed as means ± standard error (SE). Comparison of different treatments was performed by Student's t-test or analysis of variance (ANOVA) (for normally

distributed data), or the Mann-Whitney rank sum test (for non-normally distributed data). $p < 0.05$ was considered statistically significant.

Results

HDL-mimetic peptide, 4F, mitigates Aβ-induced deficits in secretion of human apoE from immortalized astrocytes

While our previous study confirmed the relevance of 4F-mediated effects to human physiology using human primary astrocytes (Chernick et al., 2018), the influence of apoE genotype on this phenomenon has not yet been described. Therefore, we used immortalized astrocytes generated from human apoE2, apoE3, and apoE4 targeted-replacement mice (Morikawa et al., 2005) to study the nuances of the 4F- and Aβ-mediated effects. Our studies show that 4F elevates apoE secretion in immortalized astrocytes expressing all 3 apoE genotypes, and counteracts the deficits induced by Aβ as well (Fig. 1). These data indicate that the effects of 4F and Aβ in the context of human apoE are not restricted to any particular genotype.

4F corrects apoE4 lipidation deficiency in primary astrocytes

While our initial results were encouraging, the relevance of immortalized cell lines is relatively low. In order to determine the more physiologically relevant role of 4F in human apoE secretion and lipidation, we used primary astrocytes from the same mouse lines that were used to generate the immortalized lines (Sullivan et al., 1997; Sullivan et al., 1998; Knouff et al., 1999; Moriwaka et al., 2005). In these primary astrocytes we confirmed the established lipidation pattern of human apoE (apoE2 > apoE3 > apoE4), and found that 4F elevates secretion and lipidation of all apoE isoforms (Fig 2).

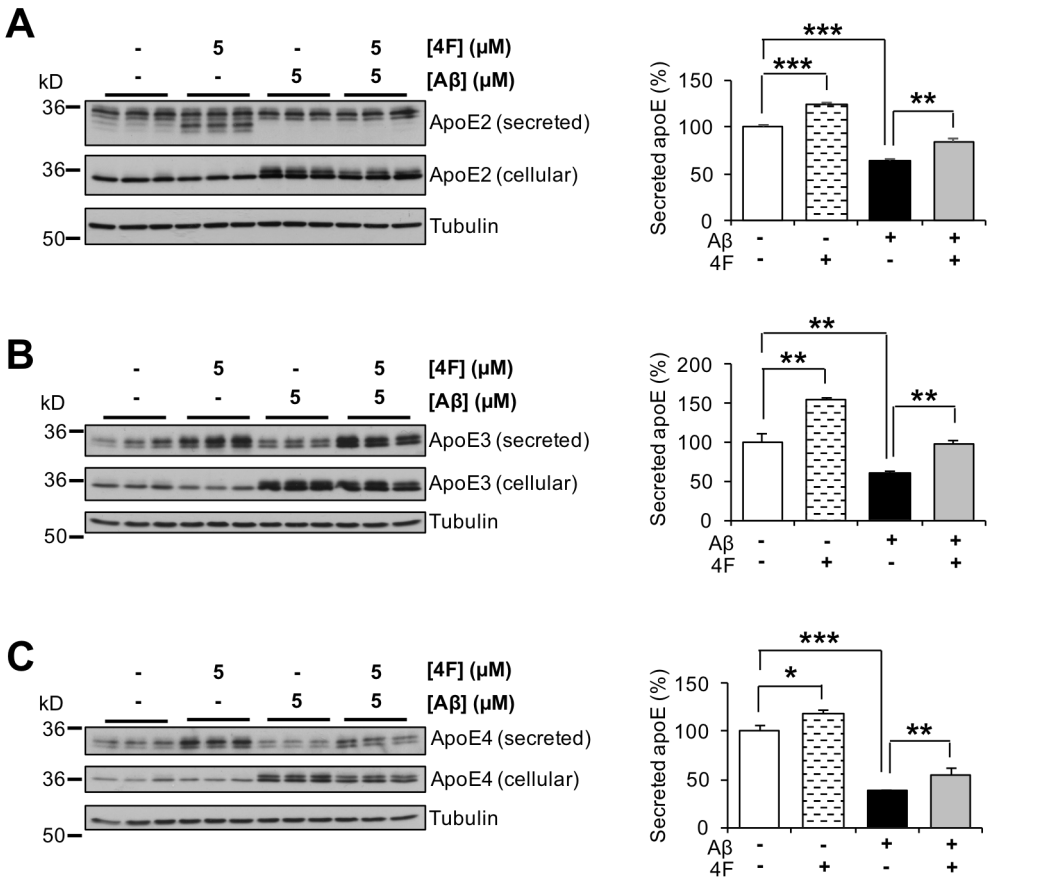

Figure 1. 4F mitigates Aβ-induced lipidation deficiency of human apoE isoforms. Immortalized astrocytes expressing human (A) apoE2, (B) apoE3, and (C) apoE4 were treated for 24 hours with or without 5μM 4F and/or 5μM Aβ in serum-free OPTIMEM. SDS-PAGE was performed on conditioned media and cell lysates. Data represent two separate experiments performed in triplicate. * = p < .05, ** = p < .01, *** = p < .001.

Importantly, 4F completely rescued the apoE4-associated deficit in lipidation, restoring it to the same level as that of apoE2 (Fig 2B).

ApoE4 is more susceptible to Aβ-induced lipidation deficiency in primary astrocytes, an effect that is rescued by co-treatment with 4F

It has previously been demonstrated that aggregated forms of Aβ, a key pathological driver of AD, reduce apoE secretion, leaving it trapped within astrocytes (LaDu *et al.* 2000, Handattu *et al.* 2013; Chernick et al., 2018). Our previous study extended this understanding to include the Aβ-mediated inhibition of apoE lipidation as well.

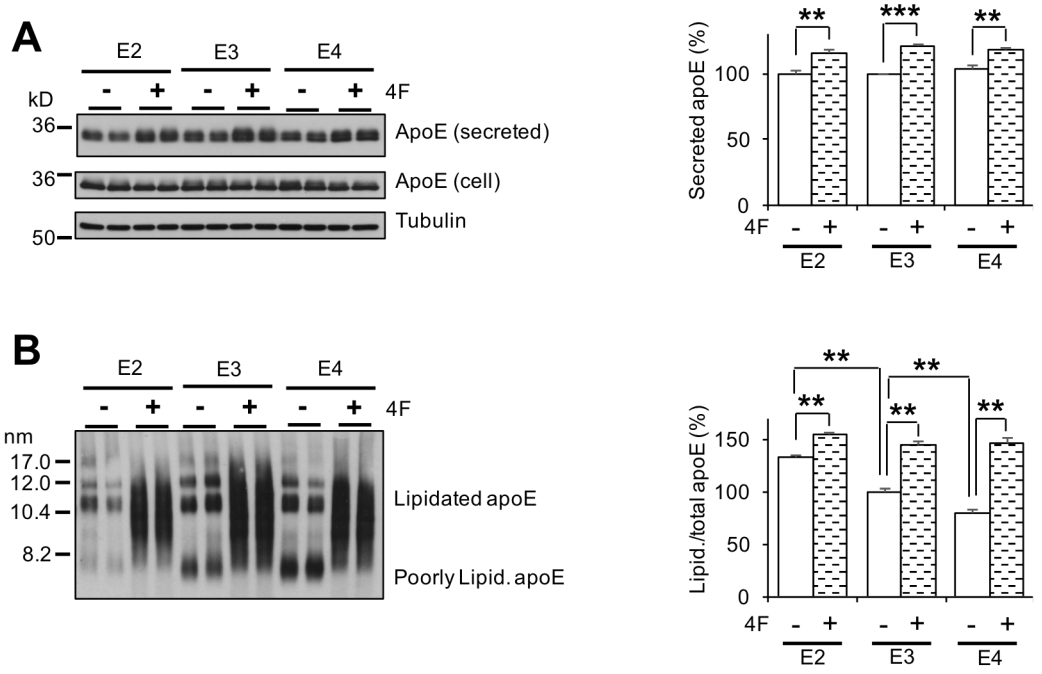

Figure 2. 4F rescues apoE4 lipidation deficiency. Murine primary astrocytes derived from human apoE2, apoE3, and apoE4 targeted replacement mice were treated for 6 hours with or without 2μM 4F in serum-free OPTIMEM. (A) SDS-PAGE and (B) NDGGE were performed on conditioned media and cell lysates. Data represent three separate experiments performed in duplicate. ** = $p < .01$, *** = $p < .001$.

Importantly, we confirmed that this phenomenon also occurs in human primary astrocytes (Chernick et al., 2018). Since apoE4 is the greatest genetic risk factor for AD, and both apoE4 and Aβ are thought to be early pathogenic drivers of AD pathology, we hypothesized that apoE isoforms may be differently susceptible to Aβ-induced detrimental effects. In order to test this hypothesis, we compared the secretion and lipidation of apoE from apoE2- and apoE4-expressing primary astrocytes upon administration of Aβ in the presence or absence of 4F.

We found that the previously described inhibitory effect of Aβ on primary astrocyte secretion and lipidation of apoE was faithfully reproduced in both apoE2 and apoE4 cells (Fig. 3). Intriguingly, however, while the effect of Aβ was equally potent in each cell type, in terms of apoE secretion, lipidation of apoE4 was reduced to a 2-fold greater extent

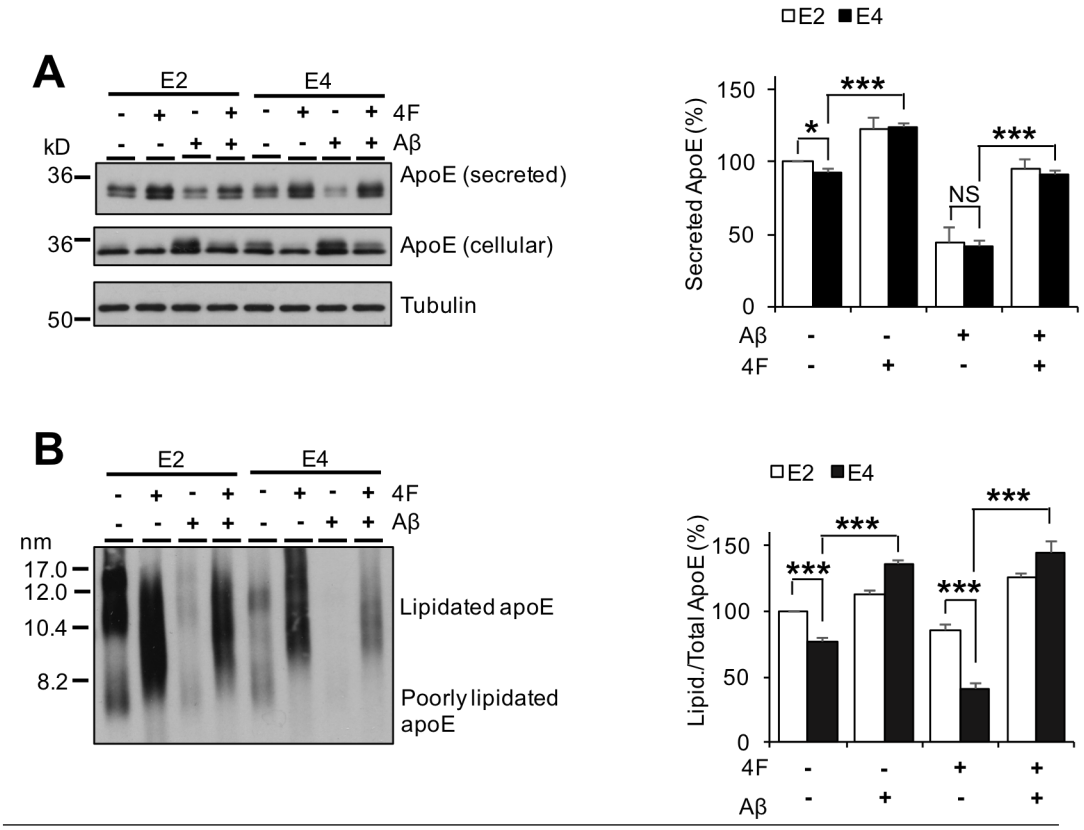

Figure 3. ApoE4 is more susceptible to Aβ-induced lipidation, an effect that is rescued by 4F. Murine primary astrocytes derived from human apoE2 and apoE4 targeted replacement mice were treated for 16 hours with or without 2μM 4F and/or 5μM Aβ in serum-free OPTIMEM. (**A**) SDS-PAGE and (**B**) NDGGE were performed on conditioned media and cell lysates. Data represent four separate experiments. ** = p < .01 *** = p < .001, NS = not significant.

than that of apoE2 (Fig. 3B). This result indicates that apoE4 is more sensitive to Aβ-induced lipidation deficiency than apoE2.

Effects of D-4F treatment on learning and memory performance of human apoE TR mice

We next sought to determine the potential of 4F to intervene in apoE4-related cognitive dysfunction *in vivo*. In order to do so, we treated 37 mice (mean age, 11.0 ± 0.2 months old), expressing apoE2 (n = 13), apoE3 (n = 12), or E4 (n = 12) with PBS (n = 18) or D-4F (n = 19) once daily, by intraperitoneal (i.p.) injection, at a dose of 2.5mg/kg body weight for 3 weeks. Body weights trended lower for all animals tested, likely due to

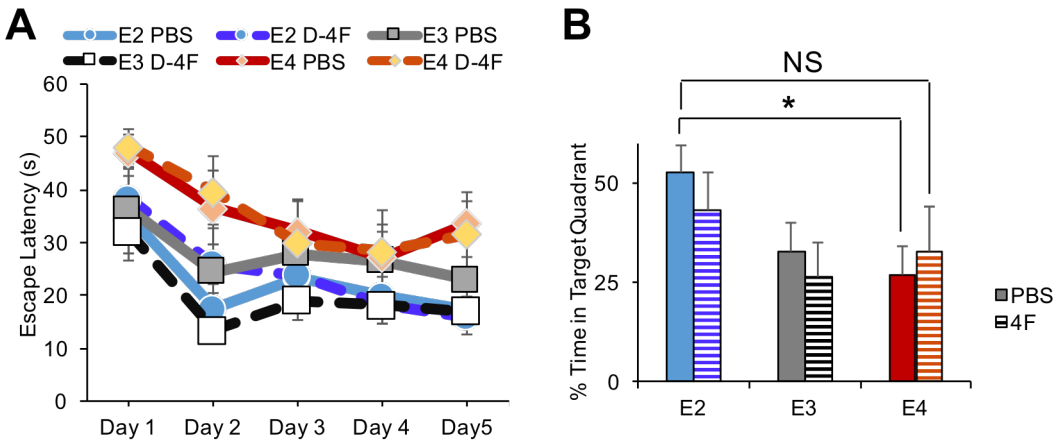

Figure 4. Effects of D-4F treatment on learning and memory performance of human apoE TR mice. Mice expressing a targeted replacement of mouse apoE for human apoE2, E3, or E4 were given D-4F or PBS daily by i.p. injection for 3 weeks. Spatial learning and memory was then tested in these mice on the Morris water maze, (A) for their ability to find a submerged platform over 5 days, and (B) for their ability to remember where that platform had been after it was removed. Total n = 3 or 4 mice per group. * = $p < .05$, NS = not significant.

stress of handling and injection, as well as novel environmental stimuli and increased activity during behavioral testing. However, no differences between PBS and D-4F-treated animals were observed. On the Morris water maze test, the animals exhibited spatial learning, with apoE2 and E3 animals learning more effectively than apoE4 animals (Fig. 4A). In order to test memory retention in these animals, they were tested in a 'probe trial' for their ability to find the target area where the escape platform had previously been placed.

During the probe trial, apoE4 animals entered the target quadrant of the pool that had previously held the platform significantly less frequently than apoE2 or E3 animals (Fig. 4B), consistent with memory deficits that have been previously described in these animals. Further analysis revealed that PBS-treated apoE2 animals spent a significantly larger percentage of the total trial time (30s) in the target quadrant of the pool than the apoE4 animals, indicating that they remembered where the platform had been during the learning acquisition phase. Conversely, apoE4 mice that received D-4F were no longer

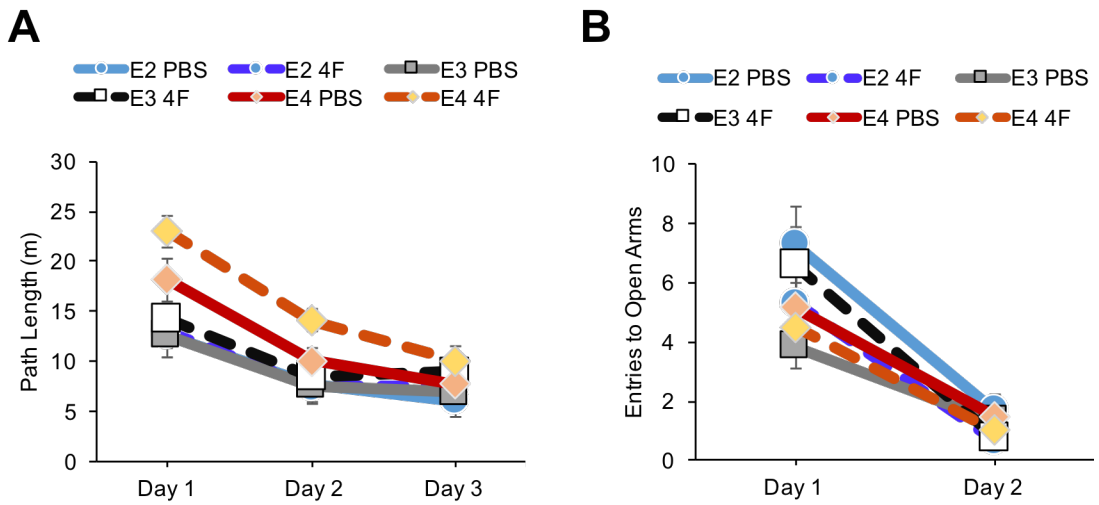

Figure 5. D-4F administration effects on locomotion and anxiety. ApoE2, apoE3, and apoE4 mice were tested for locomotor activity and anxiety by the (A) open field test and (B) elevated plus maze following administration of D-4F or PBS for 3 weeks as previously described. N = 3 or 4 per group. * = p < .05.

significantly different than apoE2 mice in their ability to find the target quadrant. the percentage of time the mice spent swimming within the target quadrant (Fig. 4B). These data indicate that D-4F abrogated apoE4-associated memory deficits in these animals. No differences were observed in tests of locomotion or anxiety (Fig. 5).

D-4F administration rescues CAA-associated memory deficits in male SwDI mice

mouse model. These mice express Swedish, Dutch, and Iowa mutations in APP, and present with high levels of amyloid deposition in the cerebral vasculature, as well as in the brain parenchyma. The cerebrovascular Aβ deposition, referred to as cerebral amyloid angiopathy (CAA), is a key pathological hallmark of AD and is found in as many as 80% of AD patients (Serrano-Pozo, et al., 2011). Due to the well-described vascular effects of 4F, as well as our previous study showing that overexpression of human apoA-I reduces CAA in AD mice (Lewis et al., 2010), we hypothesized that the HDL-mimetic

may mitigate CAA, and/or attenuate the detrimental effects on cognition that it is associated with.

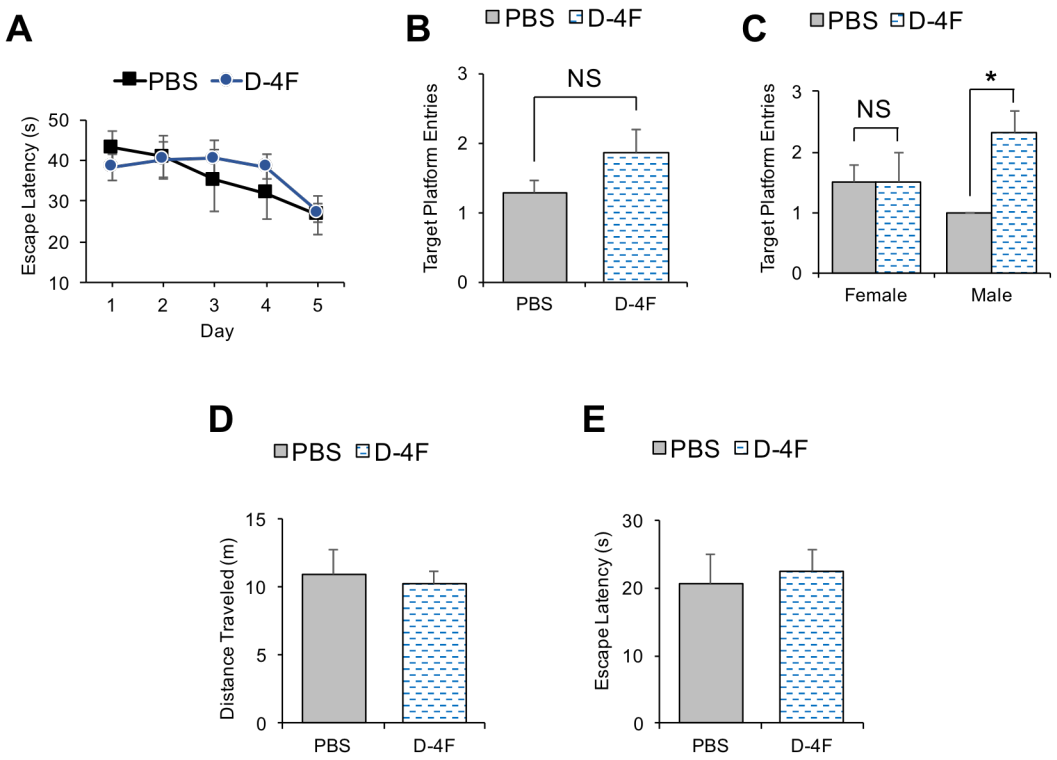

Figure 6. D-4F administration rescues CAA-associated memory deficits in male SwDI mice. Mice expressing Swedish, Dutch, and Iowa mutations in APP were given D-4F or PBS daily by i.p. injection for 3 months. Spatial learning and memory was then tested in these mice on the Morris water maze, (A) for their ability to learn to find a submerged platform over 5 days, and (B, C) for their ability to remember where that platform had been after it was removed during a probe trial. (D) The total distance traveled during the probe trial, and the escape latency to a visible platform were also determined, as controls for locomotion and eye-sight, respectively. Total n = 7 per group. * = $p < .05$, NS = not significant.

To test this hypothesis, we treated 14 SwDI mice (approximately 6 months of age at study initiation) with PBS (n = 7) or D-4F (n = 7) by daily i.p. injection for 12 weeks. D-4F has been shown to have an improved half-life over L-4F *in vivo* (Navab et al., 2005), and we have previously demonstrated that D-4F is equally effective in promoting apoE lipidation *in vitro* (Chernick et al., 2018). The mice were initially administered a dose of 5mg/kg. Body weights of the D-4F treated group were lower than those given PBS,

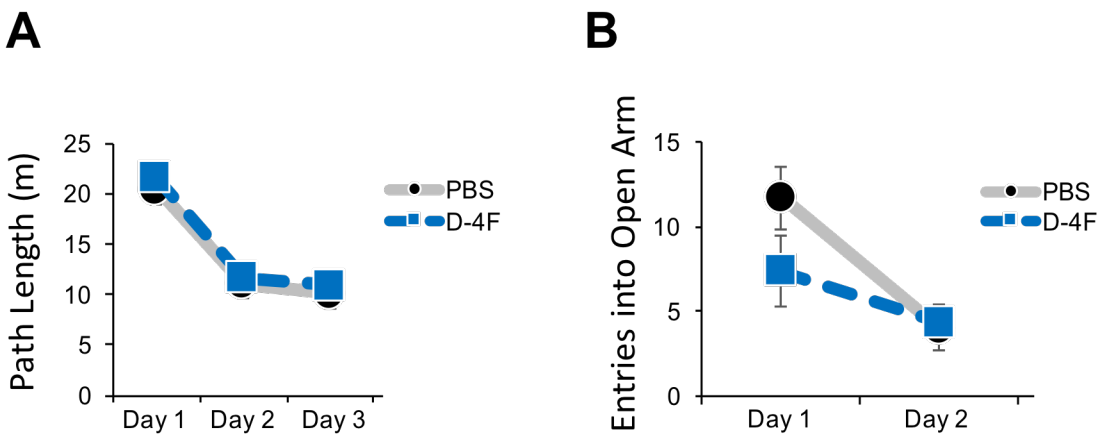

Figure 7. D-4F administration effect on locomotion and anxiety in SwDI mice. SwDI mice were tested for locomotor activity and anxiety by the (A) open field test and (B) elevated plus maze following administration of D-4F or PBS for 3 weeks as previously described. n = 7 per group: * = $p < .05$.

although no D-4F treated animals ever fell below the pre-specified limit of 80% of starting body weight. Nevertheless, we lowered the treatment dose by half, to 2.5mg/kg, after 5 weeks of treatment, and body-weights recovered in the remaining 7 weeks of the study. Whether this observed body-weight loss was due to toxicity of D-4F at the elevated dose, or elimination of fat stores due to the lipid-binding nature of D-4F and the i.p. injection occurring in immediate proximity to the fat pad each day, is unclear.

Behavioral analysis of the SwDI mice revealed no differences in learning acquisition with D-4F treatment (Fig. 6A). A trend towards increased memory retention was observed in the probe trial (Fig. 4B), although this did not reach statistical significance. Further analysis demonstrated that male SwDI mice, specifically, had dramatically improved memory retention in the D-4F group, while female mice showed no difference (Fig. 6C). No differences in swim speed or visual acuity were observed between treatment groups (Fig. 6D and 6E). No differences were observed between PBS and D-4F groups in tests

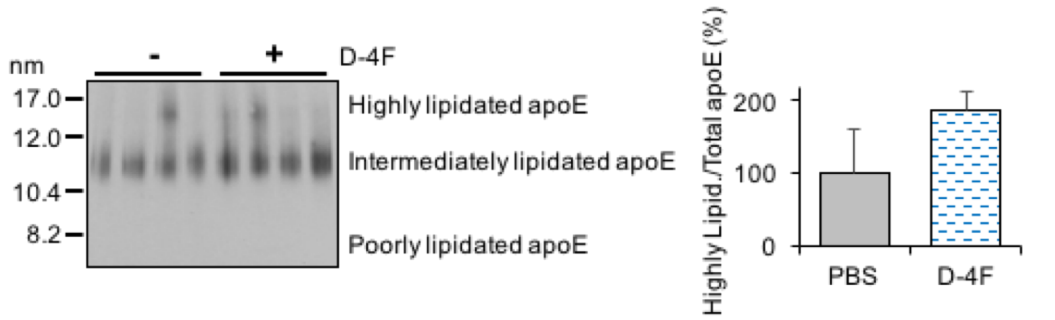

Figure 8. D-4F treatment trends toward increased apoE lipidation in the brains of SwDI mice. Following behavioral analysis, SwDI mice were sacrificed and their brains were dissected out. Flash-frozen cortical tissue was homogenized gently in PBS using a 25-gauge needle and subjected to Blue NativePAGE to determine the relative lipidation of apoE. n = 4 mice per group. NS = not significant.

of environmental habituation or anxiety (Fig. 7). These results suggest the effects of 4F treatment were not caused by any changes in non-cognitive functions in these animals.

D-4F treatment trends toward improved apoE lipidation in the brains of SwDI mice

In order to determine whether the effects of 4F that we have previously described are relevant *in vivo*, we gently homogenized flash-frozen cortical tissue from four PBS- and four D-4F-treated animals in the above study and subjected the samples to Blue NativePAGE and immunoblot analysis. The results show that apoE in the brain is, in general, more completely lipidated than *in vitro*, with virtually no signal at 8.2nm, where we typically observe poorly lipidated apoE species (Fig. 8). Nevertheless, D-4F-treated animals were found to have elevated levels of highly lipidated apoE in this study (Fig. 8A), with the appearance of a diffuse band of particles between 12 and 17nm in size, which is consistent with highly lipidated species from our previous data. This preliminary result did not reach statistical significance, owing largely to a single PBS-treated animal lying far above the mean for the vehicle group.

D-4F reduces soluble $A\beta_{42}$ levels in the brains of SwDI mice

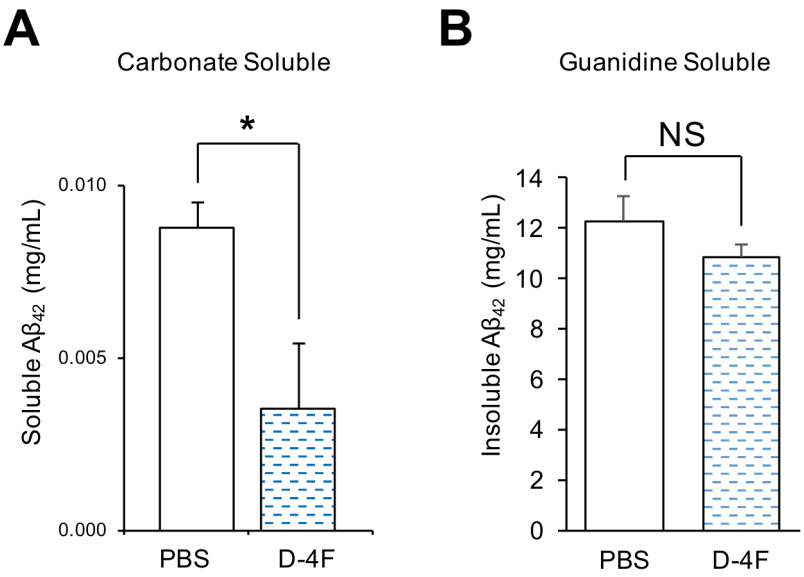

Figure 9. D-4F reduces soluble $A\beta_{42}$ levels in the brains of SwDI mice. In a separate cohort of aged SwDI mice (n = 4 per group) treated with 2.5mg/kg D-4F by i.p. for 1 week, cortical homogenates were analyzed by ELISA to determine the relative levels of carbonate soluble ("soluble") and guanidine soluble ("insoluble") Aβ in the brains of these animals. * = $p < .05$; NS = not significant.

In order to determine whether acute D-4F administration could impact amyloid pathology in the SwDI model of AD, we treated a separate cohort of 14-month old SwDI mice (n = 8 total; 4 PBS, 4 D-4F-treated) at a dose of 2.5mg/kg by i.p. injection for 1 week. Mice were then sacrificed, and flash-frozen cortical tissue was homogenized and analyzed by ELISA to determine the soluble and insoluble Aβ levels in these animals. The results from this pilot study show that D-4F treated mice have approximately 3-fold reduced levels of soluble $A\beta_{42}$ (Fig. 9A), while D-4F also produced a marginal, non-significant decrease in insoluble Aβ levels (Fig. 9B).

4F directly inhibits $A\beta_{42}$ aggregation in plastico

It had been previously established that full-length apoA-I can directly inhibit the formation of Aβ aggregates (Koldamova et al., 2001). In order to determine whether 4F illicit a

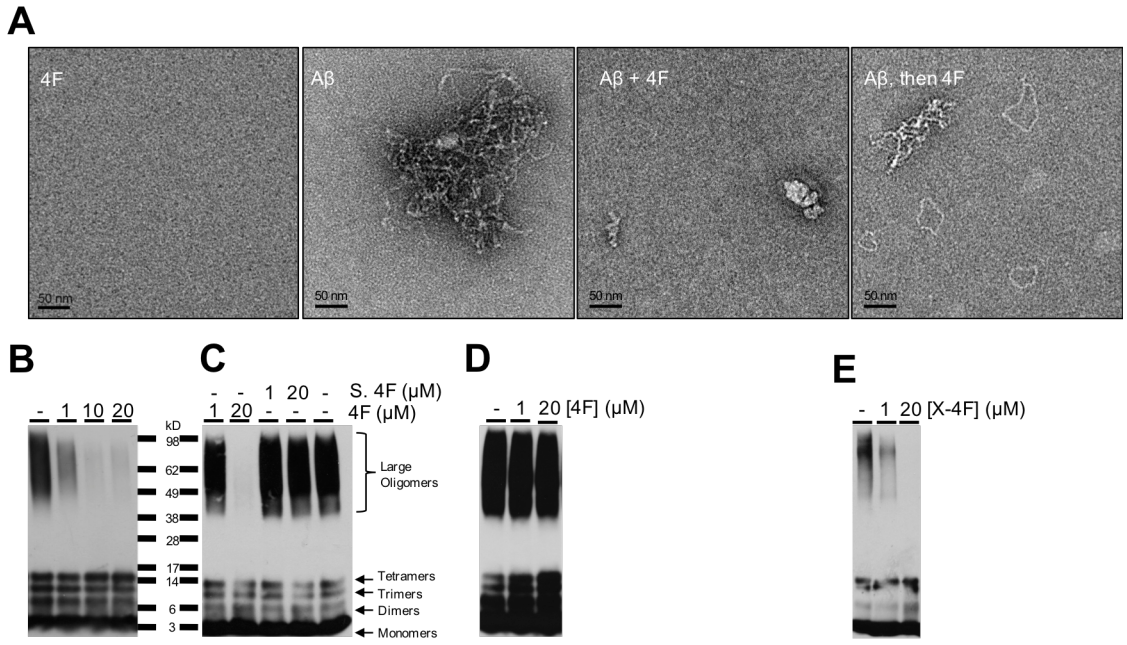

Figure 10. 4F and X-4F inhibit Aβ aggregation. (A) Representative images of TEM performed on Aβ aggregated for 24 hours with or without 4F, or aggregated without 4F for 24 hours, then allowed to aggregated for another 24 hours after addition of 4F to the solution. 4F alone was used as a control. (B) Aβ monomers were aggregated at 37°C in micro centrifuge tubes for 24 hours with or without various concentrations of 4F or (C) scrambled 4F (S. 4F). (D) As in the last panel of (A), Aβ was allowed to aggregate alone for 24 hours, then allowed to continue aggregating for another 24 hours in the presence of 4F. (E) Aβ monomers were aggregated at 37°C in micro centrifuge tubes for 24 hours with or without various concentrations of X-4F. After completion of the aggregation studies, LDS-PAGE was performed on samples using a 4-12% bis-tris gel, and transferring to a PVDF membrane, which was probed with anti-Aβ antibody 6E10.

similar effect, we aggregated Aβ as previously described in the presence or absence of 4F in the test tube. Our results indicate that 4F blocks the formation of large LDS-insoluble Aβ aggregates of sizes ranging from approximately 40 to 100 kDa (Fig. 10B). This effect occurred down to a 4F:Aβ ratio of 1:20 and was confirmed by TEM analysis (Fig. 10A, 2nd and 3rd panels). The 4F-mediated effect was not apparent with the use of a scrambled version of 4F (S. 4F), indicating that the specific sequence and amphipathic helical structure of 4F is critical to produce this effect (Fig. 10C). Interestingly, while application of 4F after 24 hours of Aβ aggregation had already occur did not appear to eliminate preformed aggregates in the same size range via LDS-PAGE analysis, TEM

showed that 4F appears to have disrupted the formation of large aggregates, while smaller aggregates remained intact (Fig 10A, compare 2nd panel with last panel). It is possible that these large aggregates, as seen by TEM, are too large to enter the LDS-PAGE gel matrix, and are composed of the smaller 40-100 kDa aggregates which are observed by immunoblot analysis.

A novel HDL-mimetic peptide, herein termed X-4F, elevates apoE4 lipidation to a greater extent than 4F

Our studies on 4F support the hypothesis that HDL-mimetic peptides may offer therapeutic benefit in AD, a hypothesis which is backed by a significant body of

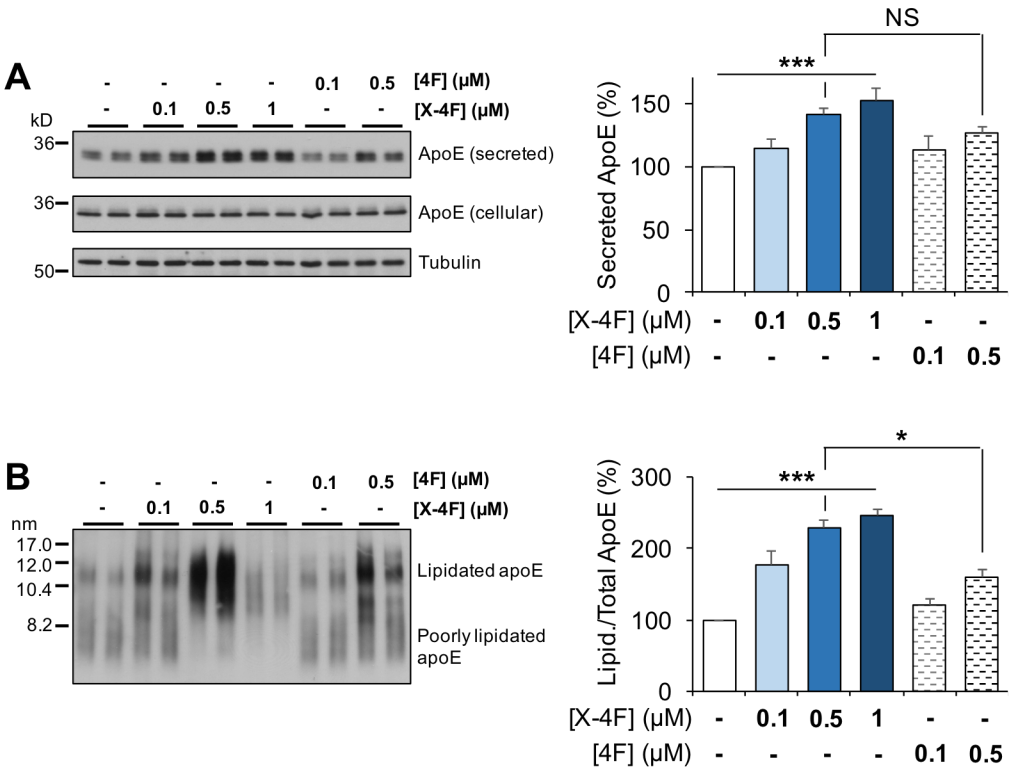

Figure 11. Novel HDL-mimetic X-4F improves apoE4 lipidation to a greater extent than 4F. Mouse primary astrocytes derived from human apoE4 targeted-replacement mice were treated for 6 hours with X-4F or 4F in serum-free OPTIMEM at a range of concentrations from 0.1 to 1μM. (**A**) SDS-PAGE and (**B**) NDGGE were performed on conditioned media and cell lysates. Data represent three separate experiments performed in duplicate. * = p < .05 ** = p < .01, *** = p < .001.

research, as described earlier in this dissertation. However, while D-4F, which we previously showed produces an equally potent effect on apoE secretion and lipidation as L-4F, increases the oral bioavailability and half-life of 4F, the concentrations at which we observe lipidation enhancement in astrocytes would be difficult to achieve in the brain. While it remains possible, based upon a large body of evidence, that systemic effects of 4F may be sufficient to reduce AD pathology and improve cognition, the blockade of detrimental Aβ-induced effects described previously would require direct targeting of astrocytes in the brain. Therefore, we modified an amino acid residue in 4F to create X-4F, with the goal of increasing its efficacy.

The novel peptide, called X-4F, was found to elevate the secretion and lipidation of apoE from apoE4-expressing primary mouse astrocytes in a dose-dependent manner (Fig 11).

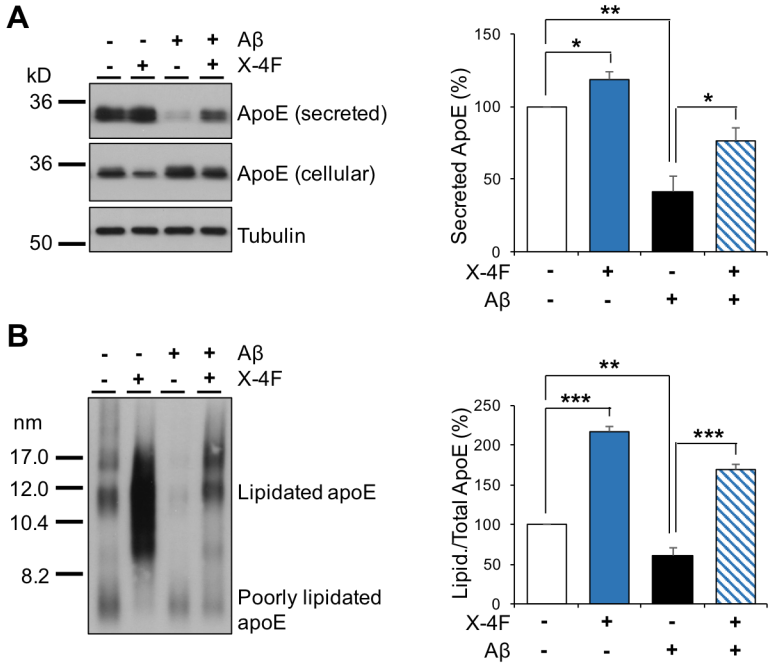

Figure 12: X-4F mitigates Aβ-induced apoE4 lipidation deficiency. Mouse primary astrocytes derived from human apoE4 targeted-replacement mice were treated for 24 hours with 0.5μM X-4F and/or 5μM Aβ in serum-free OPTIMEM. (**A**) SDS-PAGE and (**B**) NDGGE were performed on conditioned media and cell lysates. Data represent four separate experiments. * = p < .05 ** = p < .01, *** = p < .001.

The effect of X-4F on apoE4 secretion was not statistically different from that of 4F at the same concentration (Fig. 11A). Intriguingly, these preliminary data show that X-4F more than doubled apoE4 lipidation at a nanomolar concentration, 4-fold lower than the dose used in our previous studies, and was significantly more effective than 4F in this regard (Fig 11B).

X-4F rescues Aβ-induced lipidation deficiency of apoE4

We next sought to determine whether X-4F can also rescue Aβ-induced lipidation deficits at this much lower concentration. Indeed, in primary mouse astrocytes, 500nM X-4F ameliorated Aβ-induced inhibition of apoE4 secretion (Fig. 12A), and completely rescued Aβ-induced deficits in lipidation (Fig 12B).

X-4F inhibits Aβ aggregation in plastico

In order to further characterize the functional similarities of X-4F to 4F, we sought to determine whether X-4F could influence Aβ aggregation rate. Our studies indicate that X-4F faithfully recapitulates the 4F effect in this regard as well, reducing Aβ aggregation at a molar ratio of 1:20 (Fig. 10E).

Discussion

In the present study, we have shown that the HDL-mimetic peptide, 4F, mitigates apoE4 secretion and lipidation deficiency, restoring it to the same level as apoE2. These data expand upon our previous work, wherein we demonstrated that 4F increases human apoE secretion and lipidation, without identifying the influence of apoE genotype on this phenomenon. ApoE2 has been shown to be more effectively lipidated than apoE4, and is protective in AD, while apoE4 is the primary genetic risk factor for late onset AD

(Kanekiyo et al., 2014; Tai et al., 2014b; Hu et al., 2015; Hanson et al., 2013; Heinsinger et al., 2016). ApoE function and receptor binding are influenced by its lipidation state (Bu 2009; Koldamova et al., 2014), indicating that lipidation deficiency of apoE4 may underlie its pathogenic effects. It is for this reason we hypothesize that increasing apoE lipidation may provide benefit in AD patients. This hypothesis is supported by the previous findings that 4F and other HDL-mimetic peptides can attenuate cognitive impairment in mouse models of AD (described in detail in literature review 'HDL mimetic peptides as potential therapeutics in AD' section), and in one recent study, the apoE-derived peptide used (CS-6253) was shown to improve apoE lipidation *in vivo* as well (Boehm-Cagan et al., 2016).

It has been well-established that soluble aggregates of Aβ are more pathogenically relevant in AD than are insoluble plaques (Haass and Selkoe, 2007; Lesne et al., 2013; Selkoe and Hardy, 2016). Our previous work confirmed past studies demonstrating that Aβ inhibits apoE secretion from astrocytes (LaDu et al., 2000; Igbavboa et al., 2006; Handattu et al., 2013). We further expanded upon those findings to show that aggregated Aβ also reduces the lipidation of apoE in primary astrocytes from humans and mice (Chernick et al., 2018), in confirmation of a previous study that used a c-terminal fragment of apoE *in plastico* (Tamamizu-Kato et al., 2008). These effects of Aβ are seen as detrimental, as the secretion and lipidation of apoE is required for the protein to perform its canonical functions, which include lipid/cholesterol transport, synapse regeneration, immune modulation, and clearance of Aβ (Kanekiyo et al., 2014; Tai et al., 2014b; Hu et al., 2015; Hanson et al., 2013). Further, the inherent instability and incomplete lipidation of apoE4 may underlie the early stages of AD pathogenesis, as apoE4 is known to promote the aggregation Aβ (Cruchaga et al., 2012; Ma et al., 1994).

Thus, as Aβ aggregation ramps up due to other genetic and environmental risk factors, and/or due to the presence of an apoE4 allele(s), these toxic, soluble, aggregates inhibit apoE lipidation further, leading to a vicious feed-forward cycle leading to pronounced amyloid deposition in the brain and cerebral vasculature. Blocking this circular pathway with a pharmacological agent that increases apoE secretion and lipidation may slow or even halt the progression of AD.

In this study we have made the novel discovery that the detrimental effects of Aβ on apoE secretion and lipidation are influenced by apoE genotype. Compared with apoE2, apoE4 lipidation was reduced to a greater extent by the addition of Aβ aggregates to primary astrocyte culture media. This indicates that apoE is uniquely susceptible to this damaging effect, which may in part explain the elevated amyloid deposition and increased risk of AD observed in apoE4 animals and human carriers. Importantly, we show that 4F abolishes the Aβ-induced secretion and lipidation deficiency of apoE4, indicating that the HDL-mimetic may be able to block the snowball effect of Aβ aggregates and poorly lipidated apoE, and could potentially slow or even halt the progression of AD pathology, as has been demonstrated previously in the APP/PS1 mouse model of AD (Handattu et al., 2009).

Our ultimate goal is to understand the role of human apoE lipidation in the context of AD. In order to test the relevance of D-4F to the human apoE isoforms *in vivo* we treated apoE2, apoE3, and apoE4 targeted-replacement mice with PBS or D-4F. Our results show that apoE4 animals are impaired in learning and memory, as has been previously demonstrated (Bour et al., 2008; Salomon-Zimri et al., 2014), however, D-4F-treated apoE4 animals are not statistically different from PBS-treated apoE2 animals in learning or memory retention, indicating that 4F mitigates cognitive impairments in these animals.

Future analyses of flash-frozen tissue will elucidate whether D-4F injection improved lipidation of human apoE in the brains of these animals. Whether 4F can ameliorate amyloid pathology and/or cognitive impairments in mice expressing both human APP and human apoE remains to be determined.

In order to determine the relevance of our findings in animal models of AD, we performed two separate mouse studies of D-4F. The first study used the SwDI mouse model, which recapitulates CAA as a central hallmark of AD (Davis et al., 2004). We found that chronic (12-week) treatment of 6-month old SwDI animals with D-4F elevated the lipidation of mouse apoE within the brain, and attenuated memory deficits in male animals, while acute (one-week) treatment of 14-month old animals with 4F was associated with reduced soluble levels of Aβ. These data support the idea that improving lipidation of apoE can provide therapeutic benefit in AD. The specific effect on cognition in male animals, but not females, will need to be confirmed in a larger study, as sex differences are an important factor in the context of AD. Blue NativePAGE analysis of the remaining untested brains from this study will allow us a better understanding of the effect of D-4F on brain lipidation of apoE *in vivo*, as a single PBS-treated outlier appears to have strongly influenced the significance of the current results. Further, in humans, females are at an increased risk of AD (Alzheimer's Association, 2018), and whether the cognition-related benefit of 4F is truly limited to males will be important to carefully determine in future studies. However, the fact that 4F produced sex-independent effects on amyloid levels in this study is promising for the therapeutic potential of the HDL-mimetic.

Importantly, while elevating apoE lipidation may reduce amyloid aggregation indirectly, we have found that 4F may also provide a direct method of blocking the formation of

toxic aggregates. Our experiments performed *in plastico* indicate that the presence of 4F in the test tube during Aβ aggregation (as prepared for our experimental treatments) blocks the formation of a mixture of large LDS-insoluble Aβ oligomers and aggregates, of sizes ranging from approximately 40 to 100 kDa. 4F does not appear to impact the formation of small di-, tri-, and tetra-meric oligomers. It is important to note that these preliminary findings are purely observational and have not been analyzed quantitatively. There is currently significant debate as to which Aβ aggregates are the most relevant toxic species in AD. Many lines of evidence suggest the importance of soluble oligomers in driving neurotoxicity (reviewed by Mucke and Selkoe, 2012), as opposed to insoluble plaques. However, the only anti-amyloid therapeutics to show efficacy in human clinical trials, thus far, have been targeted to insoluble aggregate forms of Aβ (https://www.alzforum.org/therapeutics/aducanumab; http://investors.biogen.com/news-releases/news-release-details/eisai-and-biogen-announce-positive-topline-results-final). Two exciting recent studies indicate that only a small subset of soluble Aβ aggregates are neurotoxic (Hong et al., 2018), and also show that the anti-amyloid antibodies currently in clinical trials, including aducanumab and BAN2401, only partially protect against this toxicity (Jin et al., 2018). The ability of 4F to prohibit the formation of soluble oligomers and aggregates, and potentially to degrade larger insoluble aggregates as well, is of great interest in the context of AD treatment. Further analysis of the two SwDI cohorts included in this study by IHC and ELISA will help us to determine whether D-4F treatment reduced amyloid plaque burden in the brain parenchyma or cerebral vasculature of SwDI mice, and will offer important insights into the therapeutic potential of 4F and HDL-mimetics in general.

A large body of evidence supports a protective role of HDL and associated apolipoproteins in age-related cognitive decline and AD. A number of large-scale human clinical studies discovered that apoA-I and HDL levels are highly correlated with late-life cognitive performance, and low levels of apoA-I/HDL are associated with increased risk and severity of AD (Hottman et al. 2014). Studies in animals further support the role of apoA-I/HDL in AD (Lewis et al. 2010; Lefterov et al. 2010; Robert et al. 2016; Song et al. 2014). The potential for development of full-length HDL-associated apolipoproteins such as apoA-I or apoE as therapeutics has been studied, but is severely limited by their size, structure, and post-translational modifications. Thus, small HDL-mimetic peptides, such as 4F, with improved oral bioavailability, as well as reduced cost and difficulty of production, are attractive therapeutic candidates. Importantly, 4F has been tested in three human clinical trials in the context of cardiovascular disease. In these studies, 4F was safe and well-tolerated when administered orally or by injections, and improved the HDL anti-inflammatory index (Bloedon et al. 2008; Watson et al. 2011; Dunbar et al. 2017). Whether 4F can improve AD-related pathology or cognitive impairment in humans has not yet been studied.

We went on to show in this study that a novel HDL-mimetic peptide, X-4F, increases human apoE lipidation to a greater extent than 4F, and mitigates Aβ-induced deficits therein, at sub-micromolar concentrations. Importantly, the X-4F concentration used herein was 10-fold lower than that of Aβ, and 4-fold lower than the dose previously used to counteract Aβ effects with 4F. Conversely, while X-4F produced a numerically higher level of apoE secretion, when compared to 4F, this difference did not reach statistical significance. In light of a large body of evidence suggesting that apoE lipidation may be more important than its total level in regards to AD pathogenesis (described in detail in

Chapter 2 discussion), the more robust effect of X-4F on lipidation than secretion may be an important distinction. Whether X-4F comprised of D-amino acids produces a similar effect, or a scrambled version of the peptide fails do to so, has not yet been determined. It also remains to be studied whether X-4F can ameliorate amyloid pathology and/or cognitive decline in mouse models of AD.

This body of work, including the current study and the previous one it is an extension of, indicate that HDL-mimetic peptides can increase apoE lipidation and counteract detrimental effects of Aβ as well. These findings have significant therapeutic implications for human AD patients. Our future studies will aim to address the remaining gaps in our understanding of these agents *in vivo* in a number of animal models of AD.

CHAPTER 4 – Anti-Inflammatory Agent Minnelide Preserves Learning and Memory in a Mouse Model of Alzheimer's Disease

Introduction

Alzheimer's disease (AD), the world's leading cause of dementia among the elderly, is characterized by chronic neuroinflammation and the build-up of toxic aggregates of amyloid beta (Aβ) and hyperphosphorylated tau proteins inside the brain (Yu et al., 2014). While the etiology of AD is not fully understood, inflammatory and immune pathways are well-known to play an important role in driving AD pathological progression (Lambert et al., 2009; Mandrekar-Colucci and Landreth, 2010; Zhang et al., 2015).

Triptolide, a diterpene triepoxide derived from the Chinese herb Tripterygium wilfordii Hook F, has been shown in clinical and experimental studies to possess robust anti-inflammatory, immunosuppressive, and anti-tumor properties (Gong et al., 2008; Liu, 2011). The small molecular size and lipophilic characteristics of triptolide make it an ideal candidate for therapeutic intervention in neurological diseases (Zheng et al., 2013).

Several lines of evidence suggest that triptolide provides benefit in the context of AD. *In vitro*, triptolide increases the expression of neurotrophic markers, including nerve growth factor (NGF) and synaptophysin, reduces Aβ production, lowers presenilin expression, and inhibits Aβ-induced pro-inflammatory cytokine release (Xue et al., 2007; Jiao et al., 2008; Gong et al., 2008; Nie et al., 2012; Wang et al., 2013). *In vivo* studies further bolster the evidence supporting a neuroprotective effect of triptolide. Triptolide administration significantly delayed the onset, and reduced the severity, of experimental autoimmune encephalomyelitis (EAE) in a mouse model (Kizelsztein et al., 2009).

Further, triptolide prevented dopaminergic neuron loss, through the inhibition of microglial activation, in a rat model of parkinsonism (Gao et al., 2008).

The potential of triptolide to treat AD has been studied both *in vitro* and *in vivo*. Cell culture studies indicate that triptolide reduces oxidative stress and inhibits apoptosis induced by Aβ (Xu et al., 2016). Triptolide was also shown to protect against loss of hippocampal dendritic spine density in rats injected intracranially with Aβ (Wan et al., 2014). In the well-established APP/PS1 mouse model of AD (Jankowsky et al., 2001; Jankowsky et al., 2004), we demonstrated that triptolide improves spatial memory, mitigates neuroinflammation, and reduces amyloid deposition (Cheng et al., 2014). Our findings were supported by a number of studies, one of which also suggests that triptolide inhibits BACE-1 expression in AD mice (Wang et al., 2014; Cui et al., 2016; Li et al., 2016). Triptolide derivatives have also been studied for their potential in AD treatment (Ning et al., 2018).

Despite the potency of triptolide, the clinical application of triptolide is limited due to its poor water solubility and stability. Minnelide is a pro-drug of triptolide, designed to improve its solubility and therefore bioavailability (Banerjee and Saluja, 2015). Minnelide was created by adding a phosphate ester group to triptolide, making it water soluble. Upon entering the body, Minnelide is cleaved by phosphatases into the active agent triptolide (Chugh et al., 2012). Minnelide has been studied extensively as an anti-cancer agent (Banerjee and Saluja, 2015), however, the potential of Minnelide for the treatment of AD has not yet been explored. This study was designed to determine whether Minnelide improves memory deficit and pathological hallmarks of AD in the APP/PS1 mouse model. Our preliminary analyses indicate that Minnelide rescues memory impairment in these animals, independent of amyloid burden.

Methods

Animals and treatments

Heterozygous male APP/PS1 double Tg mice (B6C3-Tg(APPswe, PSEN1dE9)85Dbo/J; stock number 004462) (Jankowsky et al., 2001; Jankowsky et al., 2004), which develop Aβ plaques starting around 4-6 months of age, were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred with female B6C3F1/J mice (Jax Stock # 100010) to generate APP/PS1 mice (n = 11) and non-transgenic littermate controls (n = 11) for this study. APP/PS1 animals were randomly divided and administered sterile 0.9% saline (Quality Biological, Cat# 114-055-101) (n = 8) or Minnelide (n = 10) by i.p. injection once daily for 8 weeks. Minnelide was delivered at a daily dose of 100μg/kg body weight, using a 50μg/mL working solution, such that an average 50g mouse would receive a 100μL injection daily. Saline control was injected at an equal volume, as determined by each mouse's body weight. Mice were weighed weekly, starting the day prior to the start of the study, and dosing was adjusted accordingly. Non-transgenic littermates were included as normal controls. All animal procedures used for this study were prospectively reviewed and approved by the Institutional Animal Care and Use Committee of the University of Minnesota (Protocol number: 1607-33963A). All possible efforts were made to minimize animal suffering throughout the study.

Blood collection and brain tissue preparation

APP/PS1 mice and non-transgenic littermate controls were deeply anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) by i.p. injection. Complete depression of palpebral and pedal reflexes was confirmed in anesthetized mice, and then blood was collected by cardiac puncture, using heparin to preclude blood clotting. The animals

were perfused via the heart with ice-cold PBS, and brains were removed from the skull. The brains were then cut sagittally into left and right hemispheres; the left hemisphere was fixed in 4% paraformaldehyde for histological analysis, while the right hemisphere (with cerebellum and brain stem removed) was snap frozen in liquid nitrogen and stored at -80°C for further biochemical analyses.

Behavioral analysis

Three behavioral functions related to AD were assessed (spatial learning and memory, exploration of environmental stimuli, and anxiety) in this study, the methods used have been described in detail elsewhere (Lewis et al., 2010; Cheng et al., 2013; Cheng et al., 2014). The testing was performed sequentially, starting with three days of the open field test for locomotor activity, immediately followed by two days of the elevated plus-maze test for anxiety levels, and lastly, after two days without external stimulation/activity, the mice were tested for and spatial learning and memory in the Morris water maze (days 8–13). All equipment and software used in these assessments was purchased from SD Instruments (San Diego, CA).

Brain and plasma ELISA

Brain homogenates were prepared as we described previously (Cheng et al., 2013; Cheng et al., 2014). Brain $A\beta_{40}$ and $A\beta_{42}$ levels in carbonate soluble and insoluble (guanidine soluble) fractions, as well as plasma levels of $A\beta_{40}$ and $A\beta_{42}$, were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen) according to the manufacturer's protocol.

Statistical analysis:

Data were expressed as means ± standard error (SE). Comparison of different treatments was performed by one-tailed Student's t-test or analysis of variance (ANOVA). $p < .05$ was considered statistically significant.

Results

Minnelide treatment improves learning and memory retention in APP/PS1 mice

In this study we sought to determine whether Minnelide, a water soluble pro-drug of triptolide, can recapitulate the well-described beneficial properties of triptolide in improving AD-related pathology and cognitive impairment. In order to do so, we treated APP/PS1 mice (mean age, 9.0 ± 0.2 months) with 100μg/kg Minnelide (n = 10; 7 males and 3 females) daily for 8 weeks, and compared them to saline-treated APP/PS1 animals (n =8; 4 males and 4 females), as well as saline-treated wild-type (WT) non-

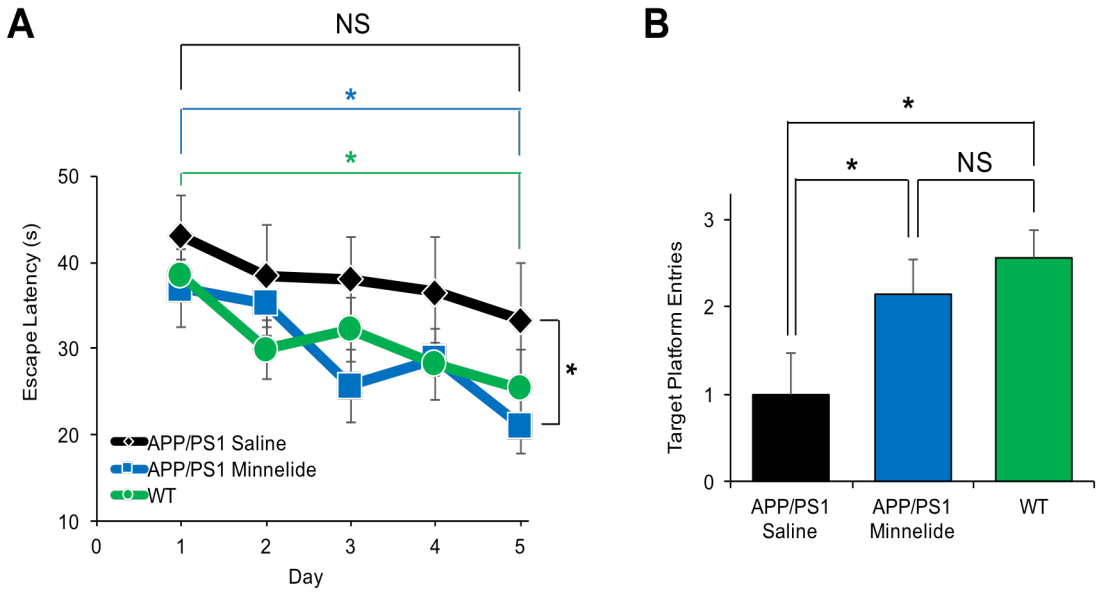

Figure 1. Minnelide treatment improves memory retention in APP/PS1 mice. Aged APP/PS1 mice were administered Saline or Minnelide daily for 8 weeks, and then (A) spatial learning (B) memory retention were tested in the Morris water maze. n = 8-11 animals per group. * = p < .05, NS = not significant.

transgenic littermates (n = 11; 8 males and 3 females) as controls. We studied the spatial learning and memory of these mice using the Morris water maze test.

During the acquisition phase, WT mice exhibited learning behavior, finding the platform significantly more quickly on the 5th day of the trial (Fig. 1A). APP/PS1 mice showed no statistically significant improvement in escape latency after 5 days of acquisition, indicating a learning deficit. The treatment of APP/PS1 mice with Minnelide restored learning capacity, and showed statistically improved escape latency on the final day of acquisition (Fig. 1A). In addition, we analyzed the intra-day differences between saline- and Minnelide-treated APP/PS1 animals. We found that Minnelide-treated mice found the target platform statistically more quickly than those given saline on days 3 and 5 of the acquisition phase, indicating an improvement in learning capacity in these animals.

On the 6th day of the water maze, the platform is removed and mice are tested for their ability to recall where it was in the days prior. WT mice circled the area where the platform was consistently, passing over the zone significantly more times than the APP/PS1 mice during a 30 second trial (Fig. 1B). Importantly, Minnelide-treated APP/PS1 mice also entered the platform zone significantly more than their saline-treated counterparts (Fig. 1B), indicating an improvement in memory retention. There was no observed difference between the WT and Minnelide groups in this study.

Minnelide treatment does not influence locomotion or anxiety in APP/PS1 mice

Behavioral assessments began with tests for motor activity in an open field, and anxiety in an elevated plus maze, prior to the Morris water maze test. No differences in activity levels were observed among the different groups of mice on days 1-3 (Fig. 2A). In addition, no differences were observed in anxiety levels among different groups in this

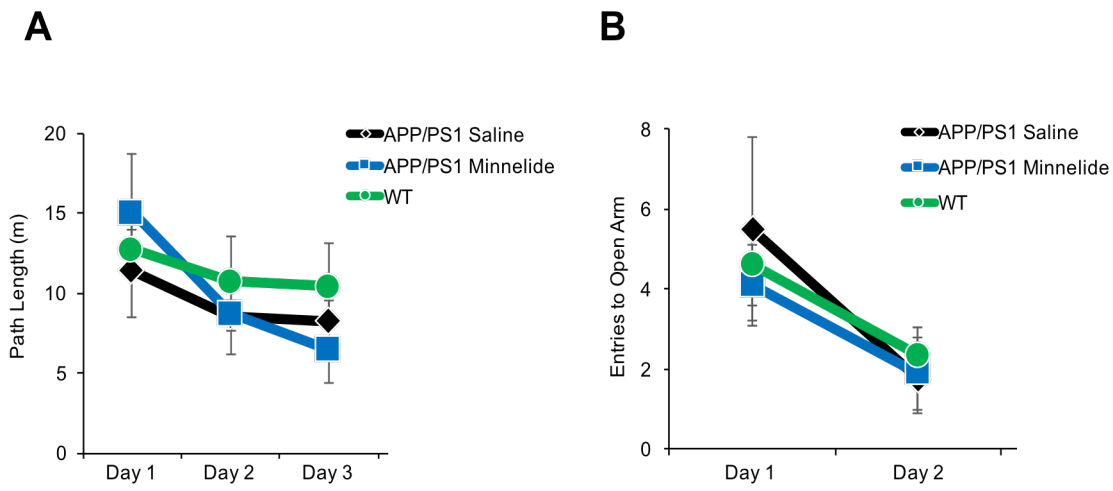

Figure 2. Minnelide treatment restores intersessional habituation in APP/PS1 mice. Aged APP/PS1 mice were administered Saline or Minnelide daily for 8 weeks, and then (A) habituation and (B) anxiety were tested in the open field test and elevated plus maze, respectively. N = 8-11 animals per group. NS = not significant.

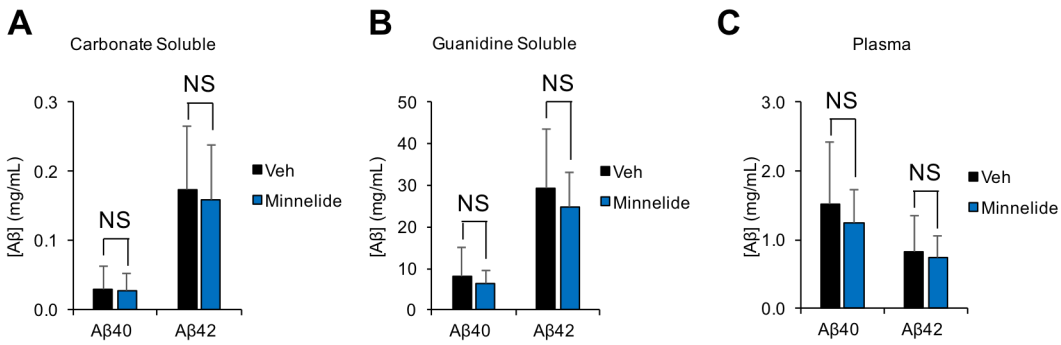

Figure 3. Minnelide treatment does not influence Aβ levels in the brains or plasma of APP/PS1 mice. APP/PS1 mice were administered Saline or Minnelide daily for 8 weeks, and then analyzed by Aβ-specific ELISA to determine the (A) carbonate soluble ("soluble"), (B) guanidine soluble ("insoluble"), and (C) plasma Aβ levels. APP/PS1-Saline, n = 8 animals; APP/PS1-Minnelide, n = 10 animals. NS = not significant.

study, as measured by the number of times the mice were willing to venture out from the enclosed arm of the plus maze apparatus into the open arm (Fig. 2B).

Minnelide had no impact on Aβ levels in the brain or plasma

Multiple previous reports have shown that triptolide reduces amyloid burden in the APP/PS1 model of AD (Cheng et al., 2014; Wang et al., 2014; Cui et al., 2016; Li et al.,

2016). We therefore sought to determine whether Minnelide recapitulates this effect in this study. Using ELISA, we found a marginal trend towards reduced levels of Aβ in the brains and plasma of APP/PS1 animals administered Minnelide, however, this was not statistically significant (Fig. 3).

Discussion

In the present study, we have demonstrated that Minnelide, a water soluble pro-drug of the anti-inflammatory agent triptolide, enhances spatial learning and memory retention in the APP/PS1 mouse model of AD. This finding has significant clinical implications, as triptolide has been shown by multiple groups to counteract amyloid pathology and improve cognition in mouse models of AD (Cheng et al., 2014; Wang et al., 2014; Wan et al., 2014; Cui et al., 2016; Xu et al., 2016). Minnelide is therefore an exciting potential anti-AD agent, with an improved pharmacokinetic profile, when compared with triptolide.

Our preliminary finding that Minnelide did not significantly impact Aβ levels in the brains of APP/PS1 animals was surprising, given the robust previous literature showing an effect of triptolide in this regard. However, this may be explained by the dosing of Minnelide. Our previous study in the same model system used 200μg/kg triptolide, whereas this study used 100μg/kg Minnelide. Approximately 30% of Minnelide is enzymatically converted to triptolide (Chugh et al., 2012). It is possible that a higher dose of Minnelide would produce an effect on Aβ deposition, however, the fact that memory deficits were attenuated in this study without requiring the alteration of Aβ levels indicates that Minnelide may mediate its beneficial effects more directly through anti-inflammatory pathways. Further immunohistochemical analysis of brain sections using antibodies against Aβ, including those specifically targeting pathogenic aggregated

forms of Aβ, will allow a more complete understanding of the level of amyloid deposition in these animals in our future work to complete this study.

In addition, immunohistochemical analysis of brain sections, as well as immunoblot analysis of brain homogenates, will allow us to gain insight into the role of gliosis, and inflammatory markers in AD animals treated with Minnelide. Based upon the previous literature, in combination with our present preliminary observation of Minnelide-induced improvement in cognition, we predict that activation of microglia and astrocytes and the expression of pro-inflammatory cytokines will be reduced in the brains of the Minnelide-treated AD mice, when compared to those given saline.

This study suggests the therapeutic potential for Minnelide in AD, owing to its improved pharmacokinetic profile over triptolide and its robust rescue of cognitive impairment in the APP/PS1 mouse model of AD. Continued analysis of the animals used in this study will allow a more complete understanding of the impact of Minnelide in AD, and may offer unique mechanistic insights as well. Whether Minnelide can provide benefit in other models of AD, or mitigate apoE4-related cognitive deficits, remains to be tested. Minnelide is currently being evaluated for safety and efficacy in ongoing and recruiting phase I and phase II open-label trials for pancreatic cancer (https://clinicaltrials.gov/ct2/show/NCT03129139; https://clinicaltrials.gov/ct2/show/NCT03117920), which are slated to conclude in December and February of 2019, respectively. Whether Minnelide may be re-purposed to provide benefit in humans with MCI or AD remains to be tested clinically.

CONCLUDING REMARKS AND PERSPECTIVES

Over the past decades, significant progress has been made in understanding the symptoms, etiology, and pathogenic mechanisms of AD. However, to date there is no effective prevention or treatment for this debilitating disease, and every year, highly anticipated clinical trials elicit redoubled disappointment. Cognitive impairment is the driving clinical outcome of this disorder, causing significant disability in sufferers and making caregiving extremely difficult, time-consuming, and costly.

Compelling evidence suggests that HDL could be an attractive target for developing therapeutic strategies to mitigate cognitive deficits in AD (Hottman et al., 2014). However, several important issues need to be addressed. The role of HDL within the brain, and particularly in the context of AD, needs to be more fully elucidated. It has been shown that HDL can be anti-inflammatory or pro-inflammatory, and it will be critical to understand what drives the lipoprotein particles to behave in one way or another, and in which contexts these actions are beneficial or detrimental. Thus, in addition to the quantity of HDL, a reliable and practical assay needs to be developed to measure the quality of HDL. We believe that apoE lipidation may be such a measurement, at least in the context of AD.

The present dissertation demonstrated that HDL-mimetic peptides, most notably 4F, can influence HDL-like particle formation in cells derived from mouse and human brains, as well as in mouse brains *in vivo*. By improving the lipidation status of apoE, HDL-mimetics mitigate deficiencies incurred by the AD risk allele apoE4, and counteract damage induced by the pathological hallmark, Aβ. These effects may halt a viscous feed-forward

cycle in its tracks, thereby attenuating a runaway train effect which would normally lead to elevated aggregation and deposition of Aβ in the brain.

This dissertation goes on to show that these HDL-mimetic peptides can improve apoE lipidation *in vivo*, which is exciting due to concerns surrounding the bioavailability, half-life, and brain penetrance of 4F. Importantly, treatment with 4F rescued memory deficits in the SwDI mouse model of AD, as well as in apoE4 targeted-replacement mice, indicating the therapeutic potential of 4F in AD. The discovery that a novel peptide, X-4F, produces more robust effects than 4F in regards to human apoE lipidation, offers the possibility that this peptide will be able to produce more robust effects on cognition, and could mitigate amyloid pathology in the brains of AD mice as well. Further studies are needed to determine whether this is the case.

Lastly, this dissertation highlights the important role of neuroinflammation in AD pathogenesis, as treatment with the anti-inflammatory agent, Minnelide, significantly improved learning and memory in the well-studied APP/PS1 mouse model of AD. The improved pharmacokinetic profile of Minnelide over its parent molecule, triptolide, makes it an enticing therapeutic option, and further studies will be needed to fully elucidate the mechanism of action and clinical potential.

It remains unclear whether HDL-mimetic peptides, or anti-inflammatory agents, must be present in the brain to exert beneficial effects relevant to AD. Further studies are needed to dissect systemic and local effects of these agents on cognitive function, and especially on vascular pathology, such as CAA. A small increase in functional HDL levels or a decrease in chronic inflammation may have a profound capacity to prevent, delay, and/or halt the progression of AD.

Aging is the greatest risk factor for AD, and our population continues to age at an alarming rate. Thus, the need for an effective therapeutic to halt the pathological progression of AD becomes greater every year. As 4F and other HDL-mimetics have already been shown to be safe in the clinical trials for cardiovascular disease, determining whether these peptides may offer therapeutic benefit in cognitive function will be of critical importance moving forward. As the role of HDL in preserving cognitive function gains an ever-greater share of the spotlight in the AD field, it is expected that HDL-enhancing therapies may provide a tangible approach to improve quality of life for millions of AD patients and their loved ones in the near future.

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