Connecting Apolipoprotein E and the Mevalonate Pathway: Insights on the Lipid Biology of Alzheimer's Disease

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Abstract

Alzheimer's Disease (AD) is a neurodegenerative disease characterized by cognitive decline and pathological accumulation of amyloid-β plaques and neurofibrillary tau tangles. The strongest genetic risk factor for development of late-onset AD is the ε4 allele of Apolipoprotein E (ApoE), which is one of three ApoE alleles found in humans next to the ε2 and ε3 alleles. ApoE is an integral component of HDL-like lipoparticles that are made and secreted predominantly by astrocytes in the adult brain in order to shuttle cholesterol, lipids, and proteins to other cells. Previously, our lab has shown that development of both amyloid and tau pathology in a mouse model of AD requires astrocyte cholesterol synthesis through the mevalonate pathway, yet whether this pathway modulates ApoE is not well-known.

The mevalonate pathway is responsible for controlling two vital cellular processes, cholesterol production and generation of substrates to be used for protein prenylation. Prenylation is a post-translational modification where an isoprenyl group is added to a protein in order to make it more lipophilic. This is particularly important for some proteins, such as the Ras superfamily of small GTPases, which require prenylation to be embedded into membranes to subsequently signal and traffic cargo throughout the cell. Chapter 1 focuses on reviewing AD, ApoE, and the different arms of the mevalonate pathway, as well as how these interact with one another. In the experiments presented in Chapter 2, we show that extracellular ApoE levels from astrocyte cultures expressing each of the three human ApoE isoforms are regulated by a form of prenylation called geranylgeranylation, and that this effect is independent of ApoE isoform. We then find that short-term inhibition of prenylation in astrocytes specifically impairs the secretion of ApoE, not its reuptake into the cell.

In Chapter 3, additional experiments and preliminary data are presented as future directions to further characterize the interactions of the mevalonate pathway and ApoE. Given the increasing interest in targeting of ApoE for treating AD, it is imperative to understand the basic biology underlying the regulation of ApoE in order to more effectively modulate this protein therapeutically, and to identify novel targets for AD intervention. Altogether, the focus of this dissertation was to better characterize the interaction between the different arms of the mevalonate pathway and ApoE in order to gain insight into potential mechanisms underlying lipid biology and AD.

Acknowledgments

The past six and a half years have been some of the hardest of my life, and I owe everything to the many people in my life who have helped get me through them.

I would first like to thank all of my past mentors who helped lay the groundwork for my passion for science. This started with my two mentors at Towson University, Dr. Paul Pistell and Dr. Chris Magalis. The latter of which whose absolute joy for research, statistics, and heavy metal was a breath of fresh air and a source of inspiration for me in my undergraduate career. I would like to also thank Dr. Brian Polster at UMB, who took a massive chance on a Psychology undergraduate student from another university and let me conduct research in an entirely new field of science. This step in particular was life changing, and I grew enormously as a scientist during this time. Thank you to Dr. Bibiana Bielekova at the NIH. I learned so much about working in a clinical setting and how to think translationally about research by working with you.

Thank you to all of my mentors at UVA who have been with me throughout all of the many project changes and course corrections. I would like to thank Dr. Zhen Yan, who took me under his wing and bolstered my knowledge and love for mitochondria. Thank you especially to my PhD mentor Dr. Heather Ferris. I know it has not been the easiest time, but it has been an incredible experience working with you and everyone in the Ferris lab the past few years. I have learned and grown so much under your guidance and I appreciate all that you have done for me. Thank you to all of my committee members, both past and present, for supporting me and giving me the push I needed to get to where I am.

I have been extremely lucky to have been able to work with so many incredible people in the Ferris lab throughout my PhD. Thank you, especially, to Drs. Josh Kulas, Thaddeus Weigel, Lisa Post, Mohsen Hosseinibarkooie, and Kayal Sankar. Your friendship, mentorship, and care for others is immense. I will never forget all of the early days and late nights, ups and downs, or gelatin fights that we shared. Thank you to the people who have supported me and my work, especially Aria Kamal and An Lo in recent years, as well as all of the students I have mentored during this time.

I would truly not have gotten through this program or been the person I am now without all of the many friends I have been so grateful to have made in Charlottesville. Between scary movies and Twin Peaks, trivia nights (go Raccoon Toe Beanz!!!), Mario Baseball and Civ5 (get bent, Alex), drinks at Rockfish, the Cane Toads documentary, friendsgiving and Halloween parties, road tripping to see the eclipse, board game nights, CCK days, and times where we just got together to laugh and talk about life, and all of the crazy things going on in the world — I am eternally grateful that we have gotten to be part of each other's lives and grow together as people. I am so proud of all of you and can't wait to see where we all go from here.

To my best friend, Juliet, thank you for being unrelentingly, unapologetically your amazing, hilarious, witty self, and the person I've sought comfort in time and time again. You are such an incredible person and I am so thankful for everything you have done for me and taught me over the last 14 years. I would never have gotten to where I am, become who I am, or grown as I have without you and your unending support, care, and wisdom. Thank you for all of the conversations, walks, vacations, laughter, music, and tears we've shared. You have completely changed my life and I would not be here without you.

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Thank you to everyone who has been part of my journey and gotten me to this point. This is as much a culmination of your work and care as it is mine. I love you all to no end.

Author's Note

At the end of this dissertation is an appendix that consists of a previously-published review I wrote with my mentor, Dr. Heather Ferris, regarding insulin signaling in the brain, its impact on mitochondrial metabolism, eating behaviors, and whole-body metabolism, as well as how it may be involved in neurodegenerative disease[44]. The note below reflects some of my personal opinions and beliefs regarding anti-fatness and food shame in science.

As a fat liberationist who has struggled immensely with disordered eating for many years and someone who has grown substantially in my perspective and truth since the publication of this paper, it is absolutely essential for me personally to take advantage of the format of this dissertation and address some aspects of this manuscript upfront.

In this paper, we use the term "obesity" and person-first language to describe people with higher BMIs. We associate eating a diet high in fat with causing harm to one's body, particularly because it leads to "obesity", metabolic disease, and potentially neurodegenerative disease. We talk about how "obesity" is a disease itself that can subsequently induce metabolic disease and even Alzheimer's Disease. We mention that "obesity" is something that needs to be treated and fixed.

<u>These are immensely harmful ways of talking about fat people and food that are deeply rooted in white supremacy, sexism, and ableism.</u> While it is true that we focus on the effects of dietary fats on brain metabolism on a molecular level and I believe there is some truth and important information to be gleamed from this, it does not change the fact that some of the ideas and language used in this manuscript perpetuate the shame

and othering that has oppressed fat people for centuries. The medical field is itself one of the biggest perpetrators of this oppression, using the same words and ideas as we did in the published review paper in this appendix. These ideas and language tell fat people that their natural bodies are wrong and need to be changed “for the sake of their own health” at ‘best’ and “so we as a society do not need to pay as much for their healthcare costs” at worst. Fat people are people, our bodies do not need fixing, and we are not a burden on society.

It is my strongly-held belief from personal experience that cellular and molecular scientists often neglect and refuse to acknowledge what are often the strongest influencing factors to a person’s health in reality: stress and shame. Stress and shame from economic and housing instability, from social and medical pressures surrounding eating and body, and from simply trying to survive in a capitalist, white supremacist, anti-fat, and ableist society. The immense stress and anxiety that come from surviving under oppressive systems significantly impacts biology, and this fact cannot be forgotten by those earnestly seeking to improve healthcare.

I do not need to go through the data demonstrating the fact that fat people are far more likely to avoid going to see a doctor when they should simply because they know they will just be told to lose weight, and that their symptoms will be ignored by healthcare professionals, as they so often are. I do not need to go through how this in itself is a massive confounding factor underlying the medical statistics comparing fat and thin people that researchers often cite as justification for their anti-fat science. I do not need to go through how molecular and cellular biology research focused on diet and exercise are intrinsically linked to anti-fatness in a world that is anti-fat and promotes anti-fat ideologies, nor how the biological cannot be separated from the sociological. I do not

need to go through the vast number of reasons why these systems are harmful and lead to far more damaging things, such as weight cycling, eating disorders, and suicide. I do not need to go through the history of the BMI, and the racism and sexism embedded in anti-fatness. There are many people far more versed in these topics than I am, and who are more poised to be authorities on these ideas than I am. They are the ones we should be listening to and learning from.

There are numerous books on these topics. Some of these that I recommend reading if you are uneasy with or disagree with what I have said in this note (or even if you do agree for that matter) are: Belly of the Beast: The Politics of Anti-Fatness as Anti-Blackness by Da'Shaun L. Harrison, Heavy by Kiese Laymon, The Body Is Not an Apology by Sonya Renee Taylor, Unshrinking by Kate Manne, What We Don't Talk About When We Talk About Fat by Aubrey Gordon, and Fearing the Black Body: The Racial Origins of Fat Phobia by Sabrina Strings. I would also recommend listening to some podcasts on these topics as well, including Maintenance Phase and Unsolicited: Fatties Talk Back.

I am leaving the manuscript unedited intentionally for posterity; to look back and reflect on personally, understand and recognize that this is where I was at the time of writing the manuscript, and to see how far I have come in my own journey with fatness, fat liberation, and food shame. I will leave this note with these words: <u>There are no bad foods. Fatness is a natural and beautiful aspect of a person's body, and it is fluid. Bodies change with time and that is normal and good. From inside the room that is body, break down the walls shutting you in.</u>

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Chapter 1. Introduction

1.1. Alzheimer's Disease

Alzheimer's Disease (AD) is a neurodegenerative disease that currently affects approximately 6.9 million people in the United States above the age of 65 and 416 million people worldwide[1,2]. It is the most common form of dementia, presenting clinically as cognitive and memory deficits, as well as changes in mood and a variety of behaviors[1]. Temporally, AD begins with an asymptomatic preclinical stage in which pathological hallmarks of the disease accumulate in the brain despite the lack of clinical symptoms[3–5]. This stage lasts for decades and is followed by a prodromal stage that is characterized by mild cognitive impairment (MCI)[3–5]. At this stage, some individuals progress to dementia, although not all will do so[3–5]. The biological mechanisms underlying the progression through the disease course and the causal factors leading to AD and dementia have not been fully elucidated despite a substantial amount of research. Here, I will introduce some of the predominant aspects of AD from pathological, genetic, and cellular perspectives.

1.1.1. Pathology

Receiving an AD diagnosis typically involves cognitive and neurological exams, brain imaging, and diagnostic tests measuring biomarkers in either the cerebrospinal fluid (CSF) or blood. Aside from this, some individuals may be diagnosed with AD post-mortem if pathological assessment of brain tissue shows evidence of the two classical hallmarks of the disease. Specifically, these hallmarks are the accumulation of amyloid-β (Aβ) plaques and neurofibrillary tau tangles. Indeed, the buildup of these peptides and proteins first identified by Dr. Alois Alzheimer in 1906 are the defining aspects of the disease that would eventually come to bear his name.

Aβ plaques are formed from the aggregation of extracellular Aβ peptides that are generated by the sequential cleavage of the amyloid precursor protein (APP) by the enzymes β-secretase and γ-secretase[6]. This process can happen either at the plasma membrane or in endosomal compartments[7–9]. These Aβ peptides accumulate when their generation outweighs their clearance from the brain either by microglial phagocytosis or the glymphatic and lymphatic systems[10]. Under these conditions, the peptides bind together and become oligomers, protofibrils, and fibrils. These aggregates then form into insoluble plaques that accumulate throughout the brain in AD, and help seed tau tangles[11–13]. These tau tangles are the product of hyperphosphorylation of the neuronal protein microtubule-associated protein tau, as excessive phosphorylation leads to self-aggregation, eventually resulting in the generation of intraneuronal tau tangles.

Staging of AD to describe disease severity for research and clinical purposes (commonly referred to as Braak staging) follows both clinical symptomology and the spread of these tau tangles throughout different regions of the brain[14]. In early stages of AD, tau tangles are often first found in the transentorhinal and entorhinal cortices, with pathology subsequently spreading into the hippocampus, and then into a variety of neocortical regions in the later stages of disease[14]. Interestingly, the presence of tau tangles, not Aβ plaques, is associated with cognitive decline[15,16]. In fact, many individuals have large amounts of Aβ built up in their brains while presenting as cognitively normal[17,18]. Nonetheless, both Aβ plaques and tau tangles have broad impacts on cellular biology that over time contribute to memory loss. Indeed, cognitive impairment is directly related to the loss of synapses and the neuron cell death that follows. The cumulative loss of neurons in the brain defines neurodegeneration, which progresses over time and occurs in large amounts in the late stages of AD. Substantial

neurodegeneration causes brain atrophy and a reduction in brain volume of AD patients. A wide array of interacting cellular mechanisms have been identified as contributing factors to the neurodegeneration in AD. Some of these, such as inflammation and alterations in lipid and mitochondrial metabolism, have garnered increasing interest and appreciation for their important contributions to AD disease progression.

1.1.2. Metabolism and inflammation

The brain utilizes approximately 20% of the body's energy, yet comprises only ~2% of total body weight. Glucose is the brain's preferred metabolic substrate, breaking it down into pyruvate through glycolysis and subsequently into acetyl-Coenzyme A (acetyl-CoA), which is used as a substrate for mitochondrial oxidative phosphorylation. Glucose and lactate metabolism are imperative for learning and memory and synaptogenesis[19–22]. AD is often referred to as a metabolic disease due to the multiple metabolic components that both contribute to disease pathogenesis and progression, and are impacted by the disease course. Mitochondrial dysfunction and neuroinflammation have been widely characterized in AD, both in mouse models and in humans[23–25]. Mitochondrial impairment may precede AD pathology in some cases and contributes to amyloid production and buildup[24,26]. Changes to mitochondrial ultrastructure and bioenergetic enzyme levels and activity were initially characterized in AD brains decades ago[24,27–35]. A number of studies have shown impaired brain glucose metabolism using fluorodeoxyglucose-positron emission tomography (FDG-PET) in AD patients compared to controls, as well as a reduced oxygen consumption rate *in vivo*[36–38].

There has long been a connection made between Type 2 Diabetes Mellitus (T2D) and AD. T2D is characterized by insulin resistance, the inability for insulin to conduct its

normal function as a result of impaired insulin receptor signaling. This may arise through a number of mechanisms, but importantly, both diseases appear to be associated with insulin resistance in the brain. Analysis of post-mortem brain slices of AD patients showed impaired insulin signaling compared to controls, even in AD patients who did not have T2D[39]. Insulin is a major metabolic regulator whose primary function is to regulate glucose homeostasis in the bloodstream. Aside from this, it has a number of effects on cellular metabolism as well, such as modulation of mitochondrial respiration and autophagy. Insulin exerts its effects throughout the body, including in the brain. Its modulation of brain metabolism leads to changes in cell function in specific locations, such as the hypothalamus, where it impacts activity and mitochondrial dynamics in hunger and satiety neurons that in turn alter liver and adipose tissue metabolism[40–44].

Because insulin is an essential regulator of mitochondrial health and function, it may be unsurprising that instances of insulin resistance coincide with dysfunctional mitochondria. As mentioned, mitochondrial abnormalities are linked with AD. When mitochondrial function is impaired, one byproduct of this is an increase in reactive oxygen species (ROS) production. Under normal conditions, ROS are neutralized by antioxidants, which in the brain are mostly produced by astrocytes. When ROS is made in excess and unable to be neutralized, oxidative stress ensues. When the cell is in a state of oxidative stress, lipids, proteins, and DNA are increasingly damaged, eventually resulting in cell death. The brain is particularly sensitive to oxidative stress due to its high energetic demand and its relatively low antioxidant capacity compared to other tissues[45–47]. As such, the oxidative stress observed in the AD brain can further contribute to neurodegeneration and disease progression.

Indeed, *in vitro* studies have suggested a role for mitochondria-derived ROS in Aβ generation[48], and oxidative stress leads to chronic neuroinflammation[22,49]. Both ROS and inflammation are known to modulate insulin signaling[44], potentially connecting these phenomena observed in AD. Further, inflammation itself can impact mitochondrial function, ROS production, and insulin signaling in both neurons and glia[23,44]. In the context of AD, neuroinflammation is characterized by the proinflammatory activation of both microglia and astrocytes, and the generation and secretion of proinflammatory cytokines from these cells. This inflammatory activation and cytokine release can become toxic to neurons and oligodendrocytes over time[50,51]. Microglia are imperative to AD biology due to their role in phagocytic uptake of extracellular Aβ through receptors such as TREM2, and their subsequent lysosomal degradation of the toxic peptide. Their ability to endocytose amyloid peptides is essential in this response and in preserving cognitive function[52]. Microglial reaction to Aβ includes their proinflammatory activation. This is a normal immunological response to any infection or insult. However, in AD, microglia adopt a disease-associated phenotype characterized by transcriptional changes that are unique to microglia under neurodegenerative conditions, and these transcriptional changes are often associated with metabolic pathways[53–57]. Specifically, alterations in microglial lipid metabolism in the cortex and hippocampus have been identified in mouse models of AD[57]. Assessing disease-associated microglia, Lee et al. (2023)[57] identified increased levels of lipid species prone to oxidation, whose oxidative products are known to induce inflammation and mitochondrial dysfunction, thus linking these mechanisms further.

Altogether, there is a strong, yet not fully elucidated link between inflammation, mitochondrial dysfunction, insulin resistance, and AD that contributes to progression of

AD. The role of insulin signaling on brain metabolism under homeostatic and disease states is the topic of Appendix 1, which is a published invited review I wrote with my mentor, Dr. Heather Ferris[44].

1.1.3. Genetic contributions to AD

Over the past few decades, there has been substantial interest in understanding what the genetic basis for the development of AD is. To this end, case studies of individual families and genome-wide association studies (GWAS) attempting to identify genetic risk factors for AD have yielded significant insight into the disease, its pathogenesis, and its progression.

AD is commonly divided into two subtypes, familial or early-onset (EOAD) and sporadic or late-onset AD (LOAD). The separation between these two categories can be attributed to their genetic components. This is because EOAD is an autosomal dominant, monogenic form of AD that leads to a particularly aggressive disease course, with high levels of Aβ accumulation at an earlier age than occurs for individuals with LOAD. As such, the genes associated with EOAD are predominantly genes involved in Aβ production, namely *APP* itself and the genes encoding the APP-cleaving proteins presenilin 1 and presenilin 2 (*PS1* and *PS2*, respectively)[58]. EOAD accounts for only 1-5% of total AD cases due to its genetic underpinnings.

The far more common LOAD is a complex, multidimensional disease that has both environmental and genetic components. While age is considered the strongest risk factor[1,59,60], GWAS have identified many gene mutations that increase AD risk. Several of these genes are involved in immune regulation, with mutations in the microglial genes *TREM2*, *PLCG2*, and *CD33* garnering much interest in recent years[61–65]. Interestingly, a large number of the identified risk factor genes encode proteins involved in lipid

metabolism, such as *ABCA7*, *CLU*, *PICALM*, *SORL1*, *SLC10A2*, and *SLC24A4*[63,65]. Of particular interest, the strongest genetic risk factor for developing AD is *APOE*, which encodes for an apolipoprotein involved in lipid transport throughout the body that is especially important in the brain[66]. Due to its strong impact on AD susceptibility, a large body of research has accumulated over the past few decades attempting to describe and understand *APOE* and the role it plays in AD.

There are three primary alleles of the *APOE* gene found in humans, *APOE2*, *APOE3*, and *APOE4*. Interestingly, humans are the only species known to have multiple *APOE* alleles[67]. *APOE4* is the ancestral allele that at some point in human history evolved into the *APOE3* allele, which itself was followed by another evolutionary mutation giving rise to the *APOE2* allele[67]. Two amino acid substitutions in the *APOE* gene are what separate these three alleles from each other. Specifically, these differences arise from the presence of either a cysteine or an arginine at amino acids 112 and 158 (*APOE2*, cys112 and cys158; *APOE3* cys112 and arg158; and *APOE4*, arg112 and arg158). Despite these seemingly small genetic differences between genotypes, their impact on the structure and function of the ApoE protein and how they influence brain physiology is striking and will be discussed in subsequent sections.

In the 1990s, a flurry of papers showed a clear influence of *APOE* genotype on AD susceptibility. It was concluded and has continued to be confirmed that *APOE4* increases risk for AD and *APOE2* confers the lowest risk, with *APOE3* between the two[68–89]. From these initial reports, it became clear that the *APOE4* allele not only increases susceptibility, but that it also leads to an earlier age of onset in a gene dose-dependent manner, even in the context of EOAD[69,90]. Further, *APOE4* increases Aβ and tau load throughout the course of AD[91,92]. A recent study utilized neuropathologically

confirmed AD cases and unimpaired non-AD controls taken from the Alzheimer's Disease Genetics Consortium to show the impact of allelic dosage and AD risk[93]. In this study, individuals with two copies of *APOE2* had odds ratios of 0.13 and 0.004 when compared to individuals homozygous for *APOE3* and *APOE4*, respectively[93]. Meanwhile, *APOE4* homozygosity led to an odds ratio of 31.22 when compared to *APOE3* homozygotes[93]. This trend showing *APOE4* homozygosity leads to increased risk for developing AD is consistent in all populations studied, although the magnitude of the risk varies based on demographics.

Population studies have found that dual carriers of the *APOE4* allele have an approximate 2-fold higher likelihood of developing AD in Hispanic and Latinx populations, and a 33-fold increased likelihood in some East Asian and Japanese cohorts[94–96]. However, recent evidence suggests that Americans of African descent are more likely to have either *APOE4* or *APOE2*, but that the risk for AD from having *APOE4* is not nearly as impactful[97,98]. Several studies have found either a smaller correlation or a lack of an association between *APOE4* and *APOE2* with Aβ plaque and tau burden in this population compared to non-Hispanic White populations[99–103]. Therefore, other genetic and environmental factors may be involved in driving AD pathogenesis in populations of people with African descent that remain to be elucidated fully. These findings underscore the fact that there is a clear need for more diverse representation of racialized groups in studies attempting to determine how *APOE* modifies AD risk, and in clinical trials for treating AD.

While *APOE4* increases susceptibility to AD in most populations and an estimated 40-60% of patients with AD are thought to have at least one copy of *APOE4*, it should be noted that this is not causal, as there are many individuals who have one or

more copy of *APOE4* that do not go on to develop AD. Additionally, there have been a few *APOE* mutations identified that appear to be protective against AD. The one that has piqued the most interest is the Christchurch mutation, which is an arginine to serine substitution at amino acid 136[104]. This mutation is rare and has thus far only been found in individuals with either *APOE2* or *APOE3*[104,105]. However, it's influence on AD pathogenesis has recently been exemplified by assessing a particular family in Colombia with about 1200 individuals that all carry the *PS1* E280A mutation[105]. While nearly all members of this group developed mild cognitive impairment (MCI) and dementia around the ages of 44 and 49 respectively, one individual who also carried two copies of the *APOE* Christchurch mutation did not develop MCI until their 70s[105]. Brain imaging analysis revealed this individual had high levels of Aβ plaque, but low levels of tau pathology and neurodegeneration[105]. Interestingly, this study also found a small number of individuals who had just one copy of the *APOE* Christchurch mutation, but this group still developed MCI at the same age as their kindred who did not have the mutation[105]. This suggests that the Christchurch mutation is highly effective in resisting autosomal dominant AD, but that this effect requires homozygosity. It is unclear exactly why this is the case, but recent reports suggest potential roles for differences in ApoE protein receptor binding, microglial activation, and responses to Aβ plaques and tau tangles[106–109]. Therefore, it is imperative to better elucidate the molecular mechanisms governing ApoE biology and the relationship between *APOE* genotype and AD risk, and these will be the topics of the following sections.

1.2. Apolipoprotein E

Given the increased understanding of the importance of *APOE* genotype on both normal brain function and in AD, a significant amount of research has focused on

elucidating the underlying mechanisms mediating these effects. In this section, I will discuss the structural and functional differences between ApoE proteins from each genotype. I will then outline the roles of ApoE in both the periphery and CNS, and how ApoE lipoparticles are thought to be generated, secreted, and recycled by cells.

1.2.1. ApoE structure and function

Apolipoproteins are responsible for modulating lipid levels throughout the body. They do this by binding to lipids, which leads to generation of lipoparticles carrying a variety of lipids and proteins, especially cholesterol. These lipoparticles shuttle this cargo to other tissues or cell types by binding to surface low-density lipoprotein receptor (LDLR) and LDLR-related protein 1 (LRP1)[110–112]. After receptor binding, lipid-laden lipoparticles are endocytosed and the cholesterol is extracted from the lipoparticle for the cell to use or degrade. Altogether, lipoproteins like ApoE are important modulators of cholesterol levels on both a whole-body and cellular level. Indication of the importance of *APOE* allele in this comes from data showing that individuals harboring the ApoE2 or ApoE4 isoforms are more likely to develop peripheral hyperlipidemia and hypertriglyceridemia, although it seems that the mechanisms for this are divergent between genotype[113,114]. Additionally, genetic deletion of ApoE is a commonly-used mouse model for atherosclerosis due to the resulting high plasma total cholesterol levels that subsequently induce inflammation[115–118]. Understanding the basic biology of ApoE is therefore critical for understanding diseases associated with cholesterol and lipid dysregulation.

Lipoprotein particles, also referred to as lipoproteins or lipoparticles, are produced in two separate pools in the body. In the periphery, the liver is the main source of lipoparticles, whereas in the brain, astrocytes are the predominant contributors to

lipoparticle generation[119,120], although both microglia and neurons may make ApoE under conditions of stress and inflammation[121–123]. Because of the size of these particles, they cannot cross the blood-brain-barrier (BBB). As such, the brain must make its own lipoparticles in order to shuttle lipids such as cholesterol between brain cell types.

The variations in the human *APOE* alleles give rise to ApoE proteins that have varied structural and biochemical properties. These differences significantly impact how the protein functions in biological systems *in vitro* and *in vivo*. Structurally, ApoE consists of a receptor-binding domain in its N-terminus, a C-terminal lipid-binding domain, and a flexible hinge region in between[66,108,124–127]. The lipid-binding capacity of ApoE depends on which isoform is expressed, with ApoE2 binding to lipids the most, followed by ApoE3 and ApoE4, respectively. The reasons for this difference are not yet very well-understood, although multiple computational and structural models have been proposed[127]. Coinciding with this idea, ApoE lipoparticles in the CSF have been found to be much smaller in individuals with ApoE4 compared to those with ApoE3 or ApoE2[128]. Nonetheless, this variability in lipidation is known to have specific effects on the conformation of ApoE that lead to differences in the N-terminal receptor-binding domain. Specifically, the increase in lipidation of ApoE2 leads to a conformational change in the hinge region of the protein that occludes the receptor-binding domain, reducing the ability for ApoE2 to bind to its receptors[126]. Therefore, the isoform difference in lipidation is opposite to the isoform difference in receptor binding, with ApoE4 binding the strongest to its receptors. Interestingly, the aforementioned *APOE* Christchurch mutation occurs in the lipid-binding domain and some reports suggest that this causes significantly lower binding affinity of this ApoE mutant to LDL receptors[108,109]. These data also coincide with an established isoform difference when it comes to the amount of

ApoE that is secreted from cells, as well as how much ApoE protein is found in the brain parenchyma and the CSF, with ApoE2 having the highest amount, followed by ApoE3 and ApoE4, respectively[66,120,129,130]. These factors, lipidation, secretion, and receptor-binding affinity, contribute significantly to the broader function of ApoE in regulating cholesterol levels. This is because these characteristics may impact the generation of the lipoparticle, how much of it is secreted from the cell, and how the lipoparticles are uptaken and recycled. These are the key determining aspects of ApoE biology that give rise to its importance in fine-tuning cellular function.

1.2.2. Lipoparticle generation, secretion, and recycling

There are multiple different types of lipoparticles that each have unique properties and compositions, and these different lipoparticles are distinguished from each other based on their density. There is significantly more diversity in peripheral lipoparticles compared to the CNS lipoparticles, with five types in the periphery that are made and secreted by the liver and the intestines[113,131]. From largest in size (least dense) to smallest (most dense), the five types of peripheral lipoparticles are chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), LDL, and high-density lipoprotein (HDL). These lipoparticles are associated with multiple apolipoproteins, which greatly impact the function of the lipoparticle (reviewed in great detail elsewhere[131–134]). Peripheral lipoparticles contain various combinations of ApoA, ApoB, ApoC, and ApoE throughout the different stages of lipoprotein metabolism that generate and modulate the particles[132,134–136]. In contrast, the brain almost exclusively has lipoparticles that resemble peripheral HDL, which is why brain lipoparticles are often referred to as HDL-like. While the brain does have some ApoA-I, ApoA-II, ApoA-IV, ApoD, ApoH, and ApoJ[137,138], its most prominent apolipoprotein is by far ApoE.

The process leading to ApoE lipidation, particle formation, and secretion is complex (Figure 1.1). After ApoE protein is translated, it gets shuttled to the endoplasmic reticulum (ER) lumen[139]. It is then directed to the Golgi apparatus and trans-Golgi network where it is glycosylated in its N-terminus, C-terminus, and hinge region[140–145]. Glycosylated ApoE is subsequently trafficked to the plasma membrane for secretion by exocytosis. Exocytosed ApoE binds to heparan sulfate proteoglycans (HSPGs) on the extracellular surface of the plasma membrane. While there is evidence suggesting small amounts of ApoE lipidation in the ER lumen[146,147], ApoE is primarily lipidated by the ATP-binding cassette (ABC) transporters ABCA1 and ABCG1, which transfer cholesterol and phospholipids to ApoE[148–153]. Interestingly, despite the differences in lipidation and size of ApoE lipoparticles from each ApoE isoform *in vitro* and *in vivo*, ABC transporters bind to each ApoE isoform to the same extent[154], suggesting this is not a factor in these lipidation differences. ABC-mediated lipidation leads to the generation of mature ApoE lipoparticles composed of free cholesterol, phosphatidylcholine, phosphatidylethanolamine, and a core of cholesterol esters[155]. *In vitro* studies of astrocytes expressing ApoE3 suggest that newly-synthesized particles are discoidal in shape and have lower amounts of neutral lipids like triglycerides and cholesterol esters compared to CSF lipoparticles[156]. These lipids appear to be generated by the enzyme lecithin-cholesterol acyltransferase, which esterifies free cholesterol in the particle, thereby leading to the particle becoming a more spherical shape resembling the lipoparticles isolated from CSF[147,157].

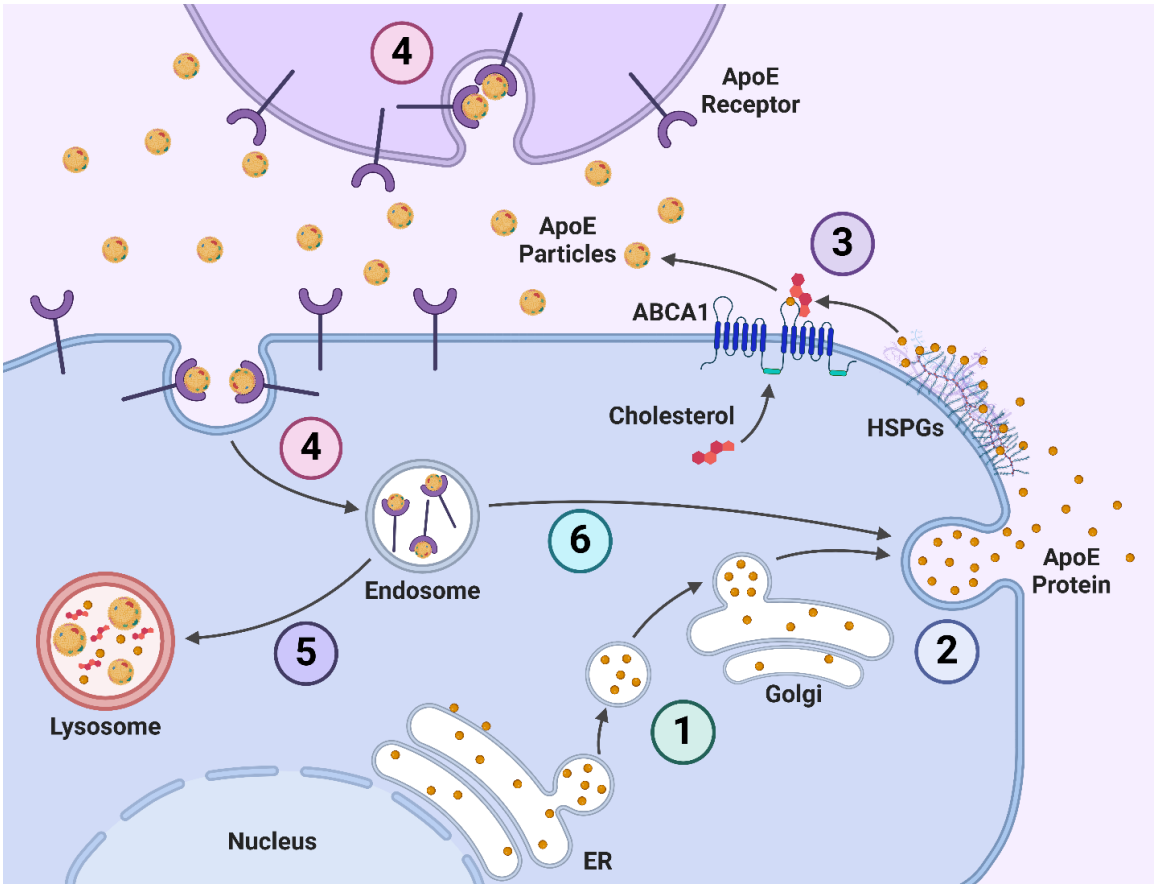


Figure 1.1. ApoE lipoparticle dynamics. (1) Newly-synthesized lipid-poor ApoE is imported into the endoplasmic reticulum (ER), where it gets processed. It is then transported to the Golgi via vesicles budding from the ER. **(2)** After being glycosylated in the Golgi, ApoE is trafficked to the plasma membrane by vesicles and secreted from the cell. **(3)** The lipid-poor ApoE protein binds to heparan sulfate proteoglycans (HSPGs) on the cell surface that give it a scaffold to bind to. The lipid transporters ABCA1 and ABCG1 add cholesterol and other lipids to ApoE, which generates ApoE lipoparticles. **(4)** These ApoE lipoparticles released into the extracellular space bind to LDLR family members on other cells or on the cell that secreted the ApoE. Uptake of ApoE lipoparticles then ensues via receptor-mediated endocytosis. **(5)** Endocytosed ApoE particles can then be transported to late endosomes followed by lysosomes to be degraded. **(6)** Alternatively, ApoE lipoparticles in early endosomes can be recycled and re-secreted from the cell. Figure made in BioRender.

Following ApoE lipidation and lipoparticle maturation, the particle is released into the extracellular space and binds to its receptors LDLR, VLDLR, LRP1, and ApoE

receptor 2 (ApoER2) on target cells. LRP1 and LDLR are the primary receptors for ApoE in the brain, although there are multiple papers showing the importance of other receptors to brain health and function through their influence on cell signaling cascades, not cholesterol modulation[158–160]. The importance of LRP1 and LDLR to brain ApoE regulation has been shown by knockout studies of both receptors leading to increased brain ApoE levels and overexpression experiments reducing ApoE content[161–165]. ApoE binding to its receptor initiates endocytosis (Figure 1.1). The molecular mechanisms for endocytosis have been studied predominantly in peripheral cells and appear to be different depending on which receptor it is binding to. For example, binding to LDLR on hepatocytes leads to endocytosis via adaptor protein-mediated clustering of the receptor with clathrin-coated pits and subsequent sorting into early endosomes[166–168]. ApoE gets released from LDLR, the latter of which is recycled back to the plasma membrane[169–171]. Binding of lipoparticles to LRP1, however, leads to internalization and sequestration to specialized compartments through interactions with different adaptor proteins partly based on the phosphorylation state of the cytoplasmic tail of the receptor[172,173]

After receptor binding and endocytosis, the particle can undergo two distinct fates (Figure 1.1). In the first, ApoE lipoparticles are broken down through the endolysosomal system, allowing for its lipid contents to be used in compartments that need them[147,174]. This promotes lipid homeostasis and also provides a pool of lipid for cells that are less capable or unable to generate their own cholesterol or other necessary lipids, like neurons. The second fate that endocytosed ApoE lipoparticles can undergo is their trafficking to intracellular compartments for either storage or re-secretion (Figure 1.1). This process has primarily been studied in peripheral macrophages and

hepatocytes[175], but some evidence suggests this also occurs in astrocytes and neurons[176].

In a variety of peripheral cell types, it was originally found that VLDL- and triglyceride-rich lipoprotein (TRL)-derived ApoE, ApoC, and lipoprotein lipase are trafficked to widely-distributed cellular compartments and subsequently back to the plasma membrane to be re-secreted, whereas the lipids from the particles, as well as other apolipoproteins like ApoB were directly shuttled to lysosomes[177–182]. Further exploration of this found that recycling of ApoE also occurs *in vivo* by hepatocytes and macrophages, and that this occurs even in LDLR knockout (KO) mice, suggesting a role for LRP1 in this process[175,181,183,184]. These early findings showed that a large proportion of the uptaken ApoE in VLDL and TRL particles is recycled and re-secreted from cells. Subsequent studies identified a mechanism by which TRL-derived ApoE associates with cholesterol and ApoA-I in endosomes and can then be recycled and secreted in association with HDL particles, suggesting the recycled ApoE is lipidated and plays a role in peripheral HDL and cholesterol metabolism[185,186]. LRP1 may shuttle ApoE to unique early endosome compartments that contain HDL in order to build a specific pool of ApoE-containing HDL for re-secretion[187]. In murine mixed glial cultures, however, LDLR may be important for ApoE recycling as LDLR KO significantly reduced the amount of a non-lipidated form of ApoE secreted by the cells that is possibly the product of ApoE recycling[146].

1.2.3. Isoform differences

Binding of ApoE particles to the LDLR and their subsequent uptake and transport into the endosomal system have consistently shown differences between ApoE isoforms. As mentioned, ApoE2 lipoparticles in particular bind to LDLR much less efficiently,

leading to reduced endocytosis of the particle and receptor[126,188]. In contrast, studies conducted in hepatoma cells suggest that there is no difference in cell surface binding, endocytosis, or endosomal transport between ApoE3 and ApoE4 particles[189]. However, a newly-published study from Dr. Gilbert Di Paolo's group using cell lines and iPSC-derived astrocytes, microglia, and neurons showed that lipidated ApoE3 and ApoE4 particles bind similarly to LDLR on the cell surface and induce equal levels of LDLR internalization, although ApoE4 particle uptake by iPSC-derived astrocytes was significantly higher compared to ApoE3[190]. Loading ApoE particles with the peroxidation-prone polyunsaturated fatty acid CE(20:4) and assessing lipid peroxidation levels revealed that ApoE4 particles induce a significantly higher amount of lipid peroxidation in lysosomes compared to ApoE3 particles, and both of these had significantly more than ApoE2 particles, with the latter finding likely owing to reduced ApoE2 receptor binding and uptake[190]. Interestingly, *in vitro* ApoE aggregation experiments found that ApoE2 and ApoE4 particles incubated with CE(20:4) aggregate to a higher degree when at a lysosomal pH than ApoE3 particles[190]. This aggregation may be the reason for the elevated lysosomal peroxidized lipid content in cells given CE(20:4)-loaded ApoE4 particles compared to ApoE3 and ApoE2. This would suggest that while ApoE2 and ApoE4 particles are more prone to self-aggregation in the lysosome when lipidated with polyunsaturated fatty acid, the detrimental impact of this aggregation (lipid peroxidation) only occurs in the case of ApoE4 because of its increased receptor binding and internalization compared to ApoE2. Therefore, ApoE isoform and lipoparticle composition can have profound impacts on ApoE dynamics, lipid metabolism, and potentially cellular function.

Differences in ApoE receptor recycling after binding to either ApoE3 or ApoE4 have previously been identified[191–194], yet the underlying mechanisms of this have not been fully elucidated. A 2021 paper proposed a hypothesis to potentially explain these ApoE isoform differences in their trafficking and recycling, and their downstream impact on diseases like AD[169]. Through molecular modeling data, the authors suggested that ApoE4 and ApoE3 may differentially bind to the LDL-A repeats in the LDLR due to differences in pH-dependence for this interaction, which could lead to ApoE4:LDLR complexes stuck in late endosomes[169]. Interestingly, in the work just discussed from Dr. Gilbert Di Paolo's group, they confirm in multiple biological systems that lipidated ApoE4 gets trapped in the endolysosomal system due to its increased propensity for self-aggregation in low pH environments[190]. This could also impair the ability of ApoE4 to be recycled within the cell. If this is the case, it may explain the reduction in cholesterol efflux from ApoE4 cells compared to ApoE3 and ApoE2 cells[195,196], as well as the known defects in lipid metabolism and the accumulation of cholesterol and esterified cholesterol in ApoE4 cells[189]. In fibroblasts, recycling of ApoE does not depend on ABCA1 activity[189], suggesting that intracellular cholesterol transport may be reduced in ApoE4 cells as a result of this sequestration of ApoE4 particles in the endolysosomal system[190]. Despite this, some evidence from a neuronal cell line suggests that cholesterol efflux is not modulated by the difference in lipoparticle recycling between ApoE3 and ApoE4[197]. However, ApoE4 does alter the membrane localization of ABCA1, which has downstream impacts on ApoE lipidation and aggregation[196]. More studies are needed to fully elucidate how these mechanisms may influence the amount of ApoE secreted and recycled from cells. In addition, understanding how ApoE recycling dynamics between isoforms modulate cholesterol homeostasis within the cell may yield impactful findings for understanding the relationship between ApoE, cholesterol, and AD in the future.

1.2.4. ApoE between brain cell-types

ApoE has a variety of roles in the brain and one of these is to mediate intercellular lipid homeostasis. To this end, neuron-astrocyte communication via ApoE lipoparticles is essential for maintaining healthy neuronal function and brain health. Neurons do not have the same capacity for generating their own cholesterol as astrocytes, who are the main producers of cholesterol in the adult brain[198]. As such, neurons rely on astrocyte cholesterol synthesis and transport via ApoE lipoparticles in order to sustain membrane cholesterol levels, and to allow for generating and maintaining synapses[147,198–200]. Once ApoE has bound to its receptors on the neuronal cell surface, it gets endocytosed and processed as described above. However, it is thought that endocytosed ApoE is recycled in neurons and then re-secreted to be uptaken by astrocytes again. Interestingly, ApoE lipoproteins are known to be able to either deliver or remove cholesterol from membranes based on their lipidation status[201]. This is also the case for brain ApoE, as de-lipidated ApoE delivered to neurons leads to cholesterol removal from the neuronal membrane and delivered back to astrocytes[9]. This process is referred to as reverse cholesterol transport and is imperative for maintaining neuronal health and function (Figure 1.2A).

Another benefit to the ApoE-mediated neuron-astrocyte crosstalk comes from a more metabolic perspective. Due to the high energetic demands of neuronal activity and a lack of cell autonomous antioxidant production in neurons, these cells are more prone to mitochondrial dysfunction and ROS accumulation. This leads to oxidation of lipids, proteins, and DNA and can eventually lead to cell death. Neurons take advantage of the endocytosed astrocyte-derived ApoE lipoparticle by loading it with oxidized lipids to reduce the intracellular levels of these toxic lipids and possibly re-secreting it. Astrocytes

then endocytose this ApoE lipoprotein loaded with neuron-derived oxidized lipids, storing them in lipid droplets, and neutralize the uptaken ROS to prevent further damage[147,202–204] (Figure 1.2B). It is currently unclear the exact mechanisms by which this and reverse cholesterol transport occur. In particular, it is not known whether the ApoE released from neurons for astrocyte uptake is recycled ApoE that originated from astrocytes or whether it is neuron-derived ApoE under times of stress. The molecular mechanisms mediating this process too are not well-characterized. Nonetheless, it is clear that the active communication between these brain cell types via ApoE lipoparticles is essential for regulating cholesterol levels and lipid metabolism.

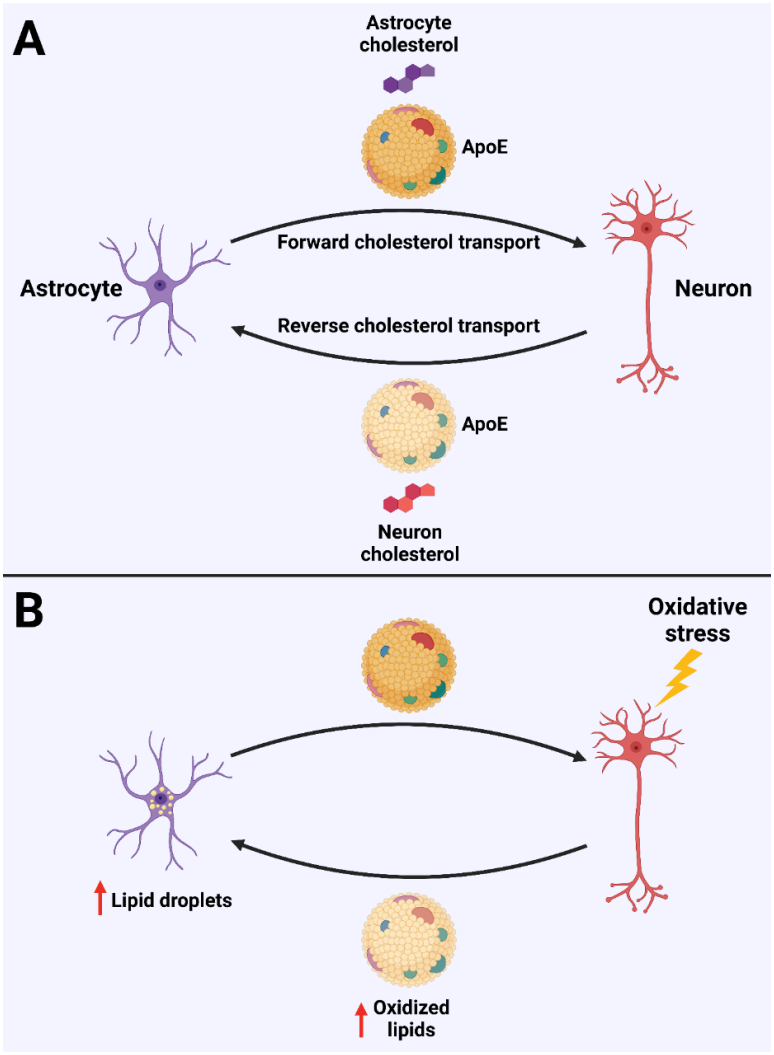


Figure 1.2. ApoE lipoparticle dynamics. (A) Astrocytes release cholesterol-rich ApoE lipoparticles for neuronal endocytosis. This process allows for the forward transport of cholesterol from astrocytes to neurons, and is critical for neuronal homeostasis. Lipid-poor ApoE lipoparticles can remove cholesterol from neuronal membranes. In addition, neurons may actively endocytose ApoE lipoparticles, load them with cholesterol, and re-secrete the particles for astrocyte uptake. This process of neuron-to-astrocyte cholesterol transport is referred to as reverse cholesterol transport. **(B)** Under conditions of oxidative stress, neurons load astrocyte-derived ApoE particles with oxidized lipids. Astrocytes uptake these particles and store the oxidized lipids in lipid droplets. Figure made in BioRender.

1.3. Lipid Metabolism

Lipid metabolism is a broad term that describes how lipids of all kinds are metabolized and catabolized in the cell. It includes their synthesis and degradation, and how these processes influence energy storage and cellular function and structure. Here, I will focus on one specific pathway that is a major regulator of lipid homeostasis within the cell, namely the mevalonate pathway. I will introduce the pathway broadly and then discuss two diverging arms of the pathway, their regulation, and biological impact.

1.3.1. Introduction to the mevalonate pathway

As the name implies, the mevalonate pathway is termed as such due to the lipid metabolite mevalonate being an initial product of the pathway that is subsequently catabolized to synthesize multiple lipid species for the cell to use. The mevalonate pathway generates a vast number of bioactive molecules, some of which include cholesterol, steroids, and non-steroidal isoprenoids. These are essential for many cellular processes, including but not limited to cell signaling cascades, cell growth and differentiation, cytoskeletal remodeling, intracellular trafficking of all kinds, and even mitochondrial respiration.

Due to its biological importance, the mevalonate pathway is highly regulated and far more complex than what will be discussed in this work. Instead, particular focus on two of the primary arms of the pathway will be given due to their role in ApoE biology, AD, and my dissertation research, as will be outlined in future sections and chapters. In order to discuss their impact on ApoE and AD, it is imperative to first have an understanding of the mevalonate pathway as a whole, and the two arms of the pathway, which separately lead to either the biosynthesis of cholesterol or the generation of isoprenoids for protein prenylation.

1.3.2. The mevalonate pathway and cholesterol

Most of the enzymes involved in cholesterol biosynthesis are located in the ER, potentially to optimize the synthetic process. The initial steps of the mevalonate pathway involve the conversion of acetyl-CoA to acetoacetyl-CoA and then to 3-hydroxy-3-methylglutaryl-CoA (HMG)-CoA, with the latter reaction conducted by HMG-CoA synthase 1 (HMGCS1). From here, HMG-CoA is used by HMG-CoA reductase (HMGCR) to produce the pathway's namesake, mevalonate. This step leading to the production of mevalonate by HMGCR is the rate-limiting step for the pathway[205]. Mevalonate is then phosphorylated by mevalonate kinase and converted to mevalonate-5-phosphate by mevalonate-5-kinase[206]. After a series of additional steps, the enzyme farnesyl diphosphate synthase makes the product farnesyl pyrophosphate (FPP). FPP serves as a branching point for the two broad arms of the mevalonate pathway because it can be used for either cholesterol synthesis or protein prenylation depending on the needs of the cell at a given moment. The importance of FPP in cellular homeostasis is broadened further by the fact that it can also undergo a chain of reactions that lead to either the synthesis of dolichol or the polyisoprene side chain of ubiquinone, with the

former being involved in the production of glycoproteins and the latter being imperative for mitochondrial metabolism and oxidative stress[207–211]. For the generation of cholesterol, however, the enzyme squalene synthase joins together two molecules of FPP in order to make squalene, and this is the first committed step of cholesterol synthesis as squalene can only be made into cholesterol or other types of sterols[205,211,212]. This then leads to the second rate-limiting step of cholesterol biosynthesis, in which squalene epoxidase oxygenates squalene to form 2,3-epoxysqualene[213]. From here, a large number of reactions occur in two branching paths that eventually lead to the synthesis of cholesterol[214].

Cholesterol itself is massively important to an array of cellular functions and processes, and to whole-body homeostasis. It is the precursor to bile acids and hormones, and vital for generating the myelin sheath that surrounds neuronal axons, as well as new dendrites and synapses[215]. Perhaps one of its most widely known roles is as an integral component of membranes, with the ability to either rigidify or fluidize membranes depending on the specific membrane composition. Its concentration varies between organelles and compartments, with particularly high levels in the plasma membrane, late secretory pathway, and some parts of the endocytic pathway[216,217]. Cholesterol can also concentrate in specific regions of a particular membrane to generate what is referred to as a lipid raft, where higher lipid content provides a scaffolding to concentrate signaling proteins in a localized manner. Taken together, it is clear that cholesterol synthesis and transport are highly regulated processes, and this regulation is essential for maintaining cholesterol and lipid homeostasis at both the cellular and organismal levels.

There is a large body of work that has focused on identifying key regulators of the cholesterol biosynthesis pathway, with much focus on the two rate-limiting steps in the pathway. How cholesterol levels are regulated, especially in regards to its synthesis, storage, exocytosis, and uptake by the cell has been another main area of attention for decades. LDLR and LRP1-mediated endocytosis of cholesterol-loaded lipoparticles, as described earlier, is one factor modulating cellular cholesterol levels. Intracellular *de novo* cholesterol biosynthesis works in tandem with cholesterol uptake and responds according to total cholesterol levels. However, both biosynthesis and uptake are themselves regulated by the master transcription factor sterol-response element binding proteins (SREBPs). There are three SREBP enzymes encoded by 2 genes, namely SREBP1a, SREBP1c, and SREBP2. SREBP1 activity leads predominantly to the synthesis of fatty acids and glucose metabolism, whereas SREBP2 is the key regulator of cholesterol synthesis[218–222]. SREBPs are membrane-bound proteins that are initially embedded in ER membranes with their C-terminal domain bound to that of the regulatory protein SREBP cleavage activating protein (SCAP)[223–226]. SCAP senses sterol levels in the cell via its sterol-sensing domain. When cellular cholesterol levels are high, SCAP keeps SREBPs in the ER, thereby stopping transcription of mevalonate pathway enzymes and reducing cholesterol synthesis[218]. In times of low cholesterol, SCAP escorts SREBPs to the Golgi via COPII vesicles that bud from the ER and fuse with the Golgi[227,228]. Here, SREBPs are cleaved sequentially by two enzymes, site-1 protease (S1P) and site-2 protease (S2P), respectively[229]. Cleavage of SREBPs by S2P releases the N-terminus of SREBP and allows for its nuclear translocation where it binds to sterol regulatory elements (SREs) within the promoter regions of SREBP target genes[218,222,230]. This activates transcription of these genes, ultimately leading to induction of cholesterol synthesis and therefore cellular cholesterol homeostasis.

In addition to transcriptional regulation by SREBPs, the enzymes in the mevalonate pathway are known to have more than 450 post-translational modifications combined that may alter their activity and degradation[214]. Indeed, ubiquitination is the most common modification and multiple E3 ubiquitin ligases are known to target enzymes involved in cholesterol biosynthesis[214,231]. Therefore, degradation of these enzymes by the ubiquitin-proteasome system is another mechanism by which cholesterol synthesis is fine-tuned for the needs of the cell. At the moment however, how impactful ubiquitination is in modulating cholesterol synthesis is not well-understood.

When intracellular cholesterol levels exceed what is needed, the cell needs to combat this. One way that it does this is by esterifying the cholesterol, which allows for the formation of lipid droplets in the cytoplasm[232,233]. Cholesterol esterification is primarily done by the enzyme ACAT1 in the ER. Lipid droplet formation is dynamic and occurs in response to cellular energetic stress. Sequestering cholesterol esters in lipid droplets allows for the cell to maintain homeostasis. Aside from exocytosis via lipoprotein particles discussed previously, an additional mechanism for combatting excess cholesterol includes the enzymatic or non-enzymatic oxidation of cholesterol, which leads to the production of oxysterols. The most prominent of which is 24(S)-hydroxycholesterol, which is able to be removed from the brain via diffusion though the blood-brain-barrier[234]. Altogether, cholesterol synthesis is an enormously complex process that serves essential functions for the body. As such, it is highly regulated through a multitude of mechanisms in order to maintain homeostasis and health on both the cellular and organismal levels.

1.3.3. Protein prenylation

Aside from cholesterol synthesis, the mevalonate pathway is critical for making the isoprenoids needed for prenylation of proteins. Prenylation is the post-translational addition of isoprenyl groups to proteins containing a prenylation sequence at the C-terminus, with a majority of the known prenylated proteins consisting of small GTPases, although nuclear lamins and the γ subunit of heterotrimeric G proteins are also known to be prenylated. This process is imperative for these proteins to function properly, as the addition of a prenyl group makes these proteins more lipophilic and therefore able to embed into membranes, as is needed for their activity. As a result, prenylation, just like cholesterol biosynthesis, is a highly complex and regulated process in order to ensure cellular homeostasis.

There are two known broad types of prenylation in mammals. The first is farnesylation, which involves the addition of the 15-carbon isoprenoid FPP to target proteins by the enzyme farnesyltransferase (FTase). FPP can also be catabolized to the 20-carbon geranylgeranyl pyrophosphate (GGPP), which is the substrate used for geranylgeranylation by the enzymes geranylgeranyltransferase type 1 (GGTase-1) and Rab geranylgeranyltransferase (GGTase-II, also known as RabGGTase). While still not well-defined, a putative GGTase-III enzyme has been described, which at the time of this work has only two identified protein substrates that it geranylgeranylates using GGPP as an isoprenoid substrate[235,236]. All four prenyltransferases are heterodimers, with both unique and shared α and β subunits. The α subunit for FTase and GGTase-1 is shared and encoded by the gene *FNTA*, whereas their β subunits differ with *FNTB* and *PGGT1B* encoding the β subunits of FTase and GGTase-1, respectively. Separately, GGTase-II

and GGTase-III are comprised of unique α subunits encoded by *RABGGTA* and *PTAR1*, respectively, and share the β subunit encoded by *RABGGTB*.

Whether a protein will be prenylated, and if so, whether it is farnesylated or geranylgeranylated, is determined by the presence of the CaaX box motif at or near the C-terminus of the protein, and what residues are present in the motif. The CaaX box motif is a short sequence of amino acids in which the C represents a cysteine, a is an aliphatic amino acid, and X is a variable amino acid. Generally, when the X residue is an alanine, serine, glycine, or methionine, the protein will be preferentially recognized by FTase and subsequently farnesylated, but if the X is a leucine, the protein may be geranylgeranylated by GGTase-1[236–238]. Interestingly, proteins with a phenylalanine in the X position may be recognized by either FTase or GGTase-1. This dual specificity is important for some proteins whose localization depends on which type of isoprenoid is added to the protein[239].

Another characteristic that may influence whether a protein is farnesylated or geranylgeranylated is whether the protein has a polybasic sequence upstream of the CaaX box motif[240]. For instance, RhoB has a polybasic sequence near its CaaX box, allowing it to be prenylated by FTase or GGTase-1. The farnesylated form of RhoB localizes predominantly to the plasma membrane, whereas its geranylgeranylated form localizes to endosomes[241]. Interestingly, the type of prenylation RhoB undergoes also alters its cellular function. Studies have shown that its farnesylated form is critical to cell growth and actin cytoskeleton modulation, as well as NF-κB activation[241–244], whereas the geranylgeranylated form is more closely associated with cell death and changes to endosome-mediated growth factor receptor recycling[239,241,245,246].

Notably, geranylgeranylated RhoB levels are strongly increased in response to FTase inhibitor treatment. Thus, while it is a protein that may be dually prenylated, it is also an example of a protein that can undergo what is referred to as cross prenylation, where a protein that is normally prenylated by either FTase or GGTase-1 can be prenylated by the other enzyme under certain circumstances, such as pharmacological inhibition. However, RhoB is not the best example of a cross prenylated protein as it may be farnesylated or geranylgeranylated under endogenous conditions. Instead, the classic example of a cross prenylated protein is K-Ras, with mutations in its K-Ras4B form being the most well-studied for its role in cancer, and N-Ras. Biochemical analyses show that K-Ras4b has a 4-9-fold higher affinity for FTase compared to GGTase-1 and that the presence of an upstream polybasic sequence (specifically composed of a series of lysine residues) contributes to this[240]. Specifically, the polybasic sequence in K-Ras4b increases its affinity for FTase, but decreases the catalytic efficiency of FTase-mediated farnesylation to similar levels as GGTase-1-mediated geranylgeranylation of the protein[240]. Indeed, under basal conditions, K-Ras is farnesylated, but in the presence of FTase inhibitors, which have been used in clinical trials for the treatment of Ras mutant-driven cancers, K-Ras can be geranylgeranylated by GGTase-1[236,247]. Kinetic studies also suggest that the hydrophobicity of the amino acid in the X position determines prenyltransferase or dual specificity of a protein[248], thereby adding further complexity to the influence of the C-terminal sequence for the prenylation of a particular protein.

Following prenylation by FTase or GGTase-1, many proteins undergo post-prenylation processing before they are shuttled to their target location in the cell and are able to conduct their biological function. Specifically, prenylated proteins are trafficked to the ER where the endopeptidase RCE1 cleaves the C-terminal peptide downstream of

the prenylated cysteine in the CaaX box motif[249]. The resulting carboxy-terminal prenylcysteine is recognized and methylated by the methyltransferase ICMT, making the C-terminus of the prenylated protein more lipophilic and able to embed into membranes in order to conduct their biological functions[250–252].

Geranylgeranylation by GGTase-II is different from that of GGTase-1 in a number of ways. For one, it is specific for Rab proteins and adds two geranylgeranyl groups to its targets. This is because many Rab proteins do not often have a CaaX box, but instead have a sequence typically containing two cysteines for the geranylgeranyl residues to bind to, such as CCXX, XXCC, or CXC, although some Rab proteins such as Rab8 and Rab13 have a CXXX sequence that gets geranylgeranylated close to their C-terminus[236,253–256]. Rab-specific geranylgeranylation is also unique in that it does not depend on C-terminal sequence, but instead requires an additional protein called REP that shuttles the Rab proteins to GGTase-II and allows for Rab prenylation to occur[257]. There are two REP proteins in mammals, REP1 and REP2. Once a Rab protein has been translated, it can bind to either of the REP proteins, and REP presents the Rab protein to GGTase-II for prenylation[254]. The α subunit of GGTase-II binds to both the β subunit and to REP, while the β subunit contains the binding and catalytic cleft that holds onto GGPP and also binds to the Rab protein. GGPP binding to GGTase-IIβ may occur prior to Rab:REP binding to the prenyltransferase in order to increase the affinity of Rab:REP with GGTase-II[258,259]. The Rab:REP complex binds through interactions of REP with GGTase-IIα and the C-terminus of Rab with the catalytic site of GGTase-IIβ. After docking, the C-terminal cysteines in the Rab protein are geranylgeranylated sequentially by two independent reactions ultimately leading to di-geranylgeranylation of the protein[258,260,261]. The addition of a single geranylgeranyl moiety to the Rab protein

reduces the affinity of the C-terminus of Rab with the GGTase-IIβ catalytic site, and singly geranylgeranylated Rabs dissociate from the prenyltransferase at this time. For the majority of Rabs, mono-geranylgeranylation does not lead to undocking and the second geranylgeranyl group addition induces undocking of the Rab C-terminus with GGTase-IIβ and re-binding with REP, with this last step causing dissociation of the Rab:REP complex[261,262]. Following dissociation from GGTase-II, REP shuttles the prenylated Rab to its target membrane for it to conduct its biological function[263,264].

Importantly, prenylation of small GTPases is not equal between protein substrates. The amount of a given protein that is prenylated versus non-prenylated can depend on a few factors and this may impact biological functions. For one, there are hundreds of proteins predicted to be prenylated, referred to as the prenylome, and only four enzymes known to conduct prenylation. Unsurprisingly, there is a significant amount of competition between potentially prenylated proteins as a result. The regulation of both isoprenoid levels and prenyltransferase expression and activity may influence this competition and modulate the prenylome as a whole[236]. In addition, how much of a specific protein is being made at a given time can modulate which proteins are being prenylated or not due to competition. It is also known that some proteins are prenylated to lower extents or slower than others[265,266]. These are only some of the factors influencing how proteins get prenylated and how much of an individual protein is prenylated out of the total pool of that protein being translated. How these factors influence the biological action of these proteins and therefore the biology and health of the cell overall is unclear. However, more research is underway to elucidate these mechanisms and their functional consequences, as well as how these processes are altered in the case of diseases such as AD. This may yield novel insight into treating a

variety of diseases, and the connection between prenylation and AD specifically will be discussed later in the following section.

1.4. Cholesterol, ApoE, and AD

As discussed, *APOE* genotype strongly influences risk for developing AD. The ApoE apolipoprotein generates lipoprotein particles in order to shuttle cholesterol around the body and between brain cell types. There is a substantial amount of evidence showing the connection between cholesterol, prenylation, intracellular trafficking, and ApoE dynamics with AD pathogenesis and disease progression. In this section, I will discuss how each of these important cellular characteristics are intertwined with each other in their relationship with AD.

1.4.1. ApoE and cholesterol in AD

ApoE interacts with many aspects of cellular biology. This is important because the different ApoE isoforms have varying impacts on cellular physiology that, in the case of ApoE4, can lead to dysfunction and generation of Aβ and tau pathology. Some studies have suggested that ApoE can bind to Aβ peptides and contribute to their toxic aggregation, and that ApoE4 binds to Aβ the strongest[267]. However, whether ApoE binds to Aβ at all *in vivo* has yet to be determined. ApoE4 stimulates the generation and secretion of amyloid to a higher extent than ApoE3 and ApoE2 in cultured human EPSC-derived neurons through activation of a signaling cascade that leads to elevated APP transcription[268]. Co-culturing WT mouse neurons with glia from ApoE targeted-replacement mice harboring each of the three human ApoE alleles and treating them with toxic Aβ oligomers showed that the presence of ApoE4 yields the highest susceptibility to neurotoxicity due to amyloid buildup[269]. Coinciding with this are data from mouse models and human AD patients that show significantly more amyloid and

tau burden in mice harboring human ApoE4[270–274], as well as human AD patients with the risk allele[91,92,94].

Given the role of ApoE in regulating lipid homeostasis and its involvement in AD pathogenesis, it is perhaps unsurprising that cholesterol has been identified as a critical regulator of AD as well. Indeed, studies in human iPSC (hiPSC)-derived glia have identified lipid metabolism as one of the most altered aspects of cells from *APOE4* carriers compared to *APOE3*[275], and *APOE4*-expressing astrocytes have an accumulation of intracellular cholesterol[276]. hiPSC-derived astrocytes from *APOE4* carriers had elevated levels of triacylglycerides and associated lipid droplet accumulation compared to cells from *APOE3* carriers[275]. ApoE4 astrocytes have impaired fatty acid oxidation compared to ApoE3 astrocytes, which also contributes to lipid droplet formation[277]. Comparing the transcriptomes of human brains of *APOE4* carriers and *APOE3* carriers revealed *APOE4* carriers had increased transcription of the mevalonate pathway genes *HMGCR*, *MVK*, and *SQLE* and decreased transcription of fatty acid metabolism genes[275]. This is in agreement with studies showing higher *de novo* cholesterol biosynthesis in *APOE4* hiPSC-derived astrocytes despite a buildup of lysosomal free cholesterol and impaired cholesterol trafficking[278]. Accumulation of neutral lipids, like cholesterol esters and triglycerides, has also been identified in post-mortem human brain tissue of individuals with AD compared to non-AD controls[279]. Interestingly, proteomic analysis of hiPSC-derived astrocytes with the three *APOE* alleles showed a reduction in cholesterol biosynthetic enzymes such as squalene synthase in *APOE4* cells despite an increase in lysosomal cholesterol accumulation[280], suggesting there may be differences between transcriptomic and proteomic data as has been described previously in multiple other fields.

Notably, excess cholesterol and lipid droplet accumulation in neurons and glia is inflammatory and can be toxic to cells over time. Levels of neuroinflammation are highly correlated with ApoE genotype. Both transcriptomic and proteomic analyses show inflammatory signaling and responses being the highest in ApoE4[273,280]. In one study, hiPSC-derived *APOE4* astrocytes even had increased inflammatory cytokine production at baseline without exogenous addition of inflammatory cytokines to initiate astrocyte reactivity[280]. Microglia secrete proinflammatory factors such as IL-1α, TNFα, and C1q that act on astrocytes and lead to a proinflammatory "reactive" astrocyte phenotype that is found in a number of diseases and in response to brain injury[50,281–283]. A seminal study in 2017 identified this cytokine cocktail for reactive astrocyte induction and showed that these activated astrocytes induce cell death of both neurons and oligodendrocytes[50]. However, it was unclear the mechanisms underlying this cytotoxic effect. This study was followed up by a group led by the first author of that 2017 paper to identify the critical factor mediating reactive astrocyte-induced cytotoxicity. Interestingly, they found that this factor was actually a long-chain saturated lipid that is carried in astrocyte-derived ApoE and ApoJ lipoparticles[51]. Genetic deletion of the enzyme that generates long-chain saturated lipids in astrocytes led to reduced lipotoxicity of astrocyte conditioned media on oligodendrocytes *in vitro* and retinal ganglion cell death *in vivo* following optic nerve crush, although toxicity was not fully abrogated suggesting this is only one contributing factor to cytotoxicity[51].

As discussed in Section 1.2.4., ApoE lipoparticles can either deliver or remove cholesterol from membranes based on their lipidation status[201]. Astrocytes are the primary producers of both ApoE and cholesterol in the adult brain and astrocyte-derived ApoE lipoparticles deliver cholesterol to neuronal membranes to support their health and

function. A study out of our lab in collaboration with the lab of Dr. Scott Hansen at The Scripps Research Institute in Florida assessed the importance of astrocyte cholesterol carried by ApoE on neuronal Aβ production[9]. Using super-resolution microscopy, it was found that astrocyte-derived ApoE delivers cholesterol to the neuronal plasma membrane, as expected[9]. This change in membrane lipid composition led to increased association of APP with the pro-amyloidogenic cleavage enzymes β- and γ-secretase in lipid rafts, thereby increasing Aβ production[9]. Interestingly, when neurons were given delipidated ApoE that removed membrane cholesterol in neurons, this association of APP with β- and γ-secretase in lipid rafts was decreased, coinciding with reduced Aβ production[9]. Therefore, cholesterol-loaded ApoE lipoparticles from astrocytes are critical modulators of amyloid production in neurons *in vitro*. To test whether this was the case *in vivo*, the authors crossed an AD mouse model that makes both pathological amyloid and tau with a mouse deficient in cholesterol synthesis specifically in astrocytes[9,284]. They found that genetic deletion of astrocyte cholesterol synthesis nearly completely abolished generation of amyloid and tau pathology in the hippocampus and cortex of these mice[9]. Further, ApoE targeted-replacement mice with ApoE4 had higher APP levels in lipid rafts compared to mice with ApoE3[9]. In an amyloidogenic mouse model of AD, they found increased APP in lipid rafts and total cholesterol levels compared to non-AD mice[9]. No ApoE isoform differences were found in lipid raft association or total cholesterol levels in the context of AD[9]. Overall, these findings strongly suggest that astrocyte cholesterol delivered on ApoE lipoparticles is an essential mediator of AD pathogenesis by modulating neuronal lipid raft dynamics, as well as APP trafficking and cleavage.

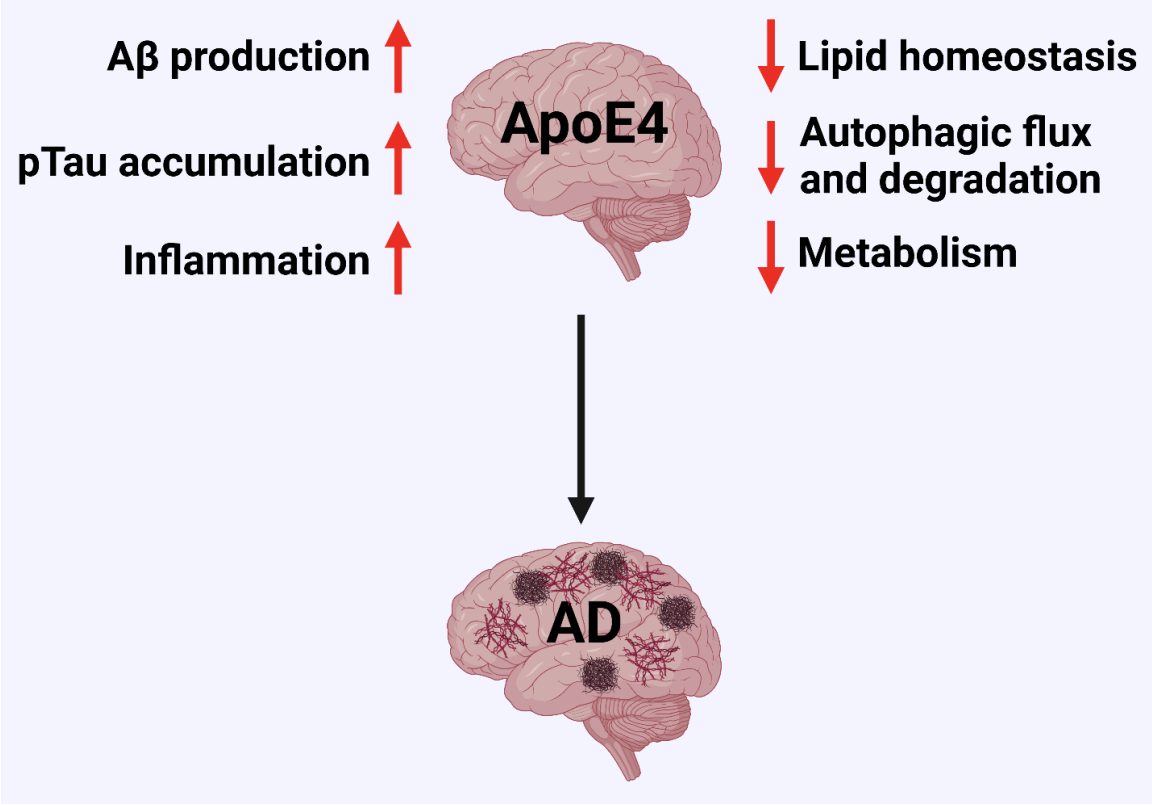


Figure 1.3. ApoE4 impairs cellular homeostasis and contributes to Alzheimer's Disease pathogenesis. ApoE4 expression is associated with several alterations in homeostatic cellular functioning. ApoE4 increases the generation of amyloid and tau pathology, leading to earlier onset and more severe disease in AD. Autophagic flux and degradation are reduced in ApoE4 brains due to impaired lysosomal acidification and intracellular trafficking of endosomes. ApoE4 also leads to an elevated inflammatory phenotype, decreased lipid homeostasis, and impaired metabolism, all contributing to AD pathogenesis and disease progression. Figure made in BioRender.

Taken together, research revealing the importance of ApoE and cholesterol in modulating AD risk and pathology has burgeoned over the past few decades. This has led to substantial interest in targeting ApoE and cholesterol metabolism for treating AD. There have thus far been multiple targets for doing this, including ApoE itself. A series of papers from the Holtzman lab have shown efficacy of reducing Aβ load and tau seeding with an antibody targeting poorly-lipidated human ApoE that is found around amyloid plaques, yielding stronger impact to amyloid levels than the FDA-approved anti-amyloid

immunotherapy aducanumab[285–287]. Others have taken advantage of the known effect of cholesterol and ApoE lipidation on AD pathology by using drugs that modulate these in AD mouse models. Specifically, increasing ABCA1 expression and lipidation of ApoE was beneficial for reducing amyloidogenesis[288]. Intravenous treatment of monkeys with the ABCA1 agonist CS-6253, however, did not change CSF Aβ levels, although sample sizes were small, a model of AD was not used, brain penetrance of the drug was low, and the duration of the study was relatively short[289]. An agonist of the nuclear hormone receptor liver X receptor (LXR), which regulates transcription of ApoE among many other genes, was also successful in increasing CSF ApoE, cholesterol, and Aβ levels, suggesting increased clearance of toxic oligomers[290]. Another approach that has garnered attention more recently is the idea of modulating the composition of ApoE lipoparticles, as it was found that inflammatory, reactive astrocytes load ApoE particles with a toxic lipid species that leads to cell death[51]. This in particular is a new idea that will be discussed in Chapter 3 of this work.

1.4.2. Prenylation, trafficking, and AD

While there has been much focus on cholesterol in AD and ApoE biology, much less of a focus has been given in regards to the role prenylation plays in AD pathogenesis or how it may be impacted during the course of the disease. Nonetheless, there are a number of lines of evidence pointing to a connection between them. For one, geranylgeranylation is important for the proper secretion of ApoE from primary murine mixed glial cultures and brain slices[291]. In addition, Kotti et al. (2008)[292] used a mouse model of mevalonate pathway deficiency to show that hippocampal long-term potentiation (LTP) is impaired in these mice and adding back a geranylgeranylation substrate was able to rescue this. Addition of the farnesylation substrate farnesol was

unable to rescue LTP, suggesting that geranylgeranylation in particular is important for hippocampal LTP and by extension learning and memory[292]. *In vivo* studies have also shown a role for prenylation in modulating AD pathology and vice versa.

In the frontal cortex of human males with AD, levels of FPP and GGPP are elevated, coinciding with transcripts of FPP synthase and FTase protein levels[293–295]. These data may suggest either impaired prenylation that induces substrate accumulation or a broader impact on isoprenoid biosynthesis by the mevalonate pathway leading to changes in substrate production and enzyme transcription as a result of AD. Corresponding with this, prenylation levels are increased in an amyloidogenic mouse model of AD compared to non-AD mice[296]. However, there is data supporting the idea that prenylation is involved in the pathogenesis of AD as well. For example, heterozygous deletion of FTase and GGTase-1 leads to reduced Aβ plaque accumulation in an AD mouse model[297]. This same study showed that FTase haploinsufficiency in an AD mouse model improved learning and memory[297]. The mechanism of this rescue has not been fully elucidated, but it may be the case that a reduction in FTase activity due to haploinsufficiency increased substrate levels for geranylgeranylation that could be used for LTP. However, this was not assessed and other mechanisms are likely at play. Another study utilizing a model of tauopathy showed that tau buildup occurs in part due to the activity of the farnesylated protein Rhes[298]. *In vitro* and *in vivo* experiments using the FTase inhibitor lonafarnib alleviated tau pathology by reducing Rhes activity, thereby initiating lysosomal activation[298]. Together, these studies show an evolving understanding of the connection between prenylation, ApoE, and AD, the full extent of which requires further investigation.

Prenylation is critical for the proper functioning of small GTPases in the Ras superfamily, including those that mediate the secretory, endocytic, and recycling pathways. As discussed previously, these are all critical pathways regulating ApoE and ApoE receptor activity. Further, astrocytes express many of these small GTPases essential for intracellular trafficking and their prenylated forms[299]. Therefore, prenylation may be important for regulating these pathways in the context of AD as well. Interestingly, LRP1 is a receptor for Aβ and interacts with APP via its cytoplasmic tail to regulate its trafficking and processing at multiple steps[148,300–302]. Waldron et al. (2008)[300] found that APP associates with LRP1 early in the secretory pathway, before they reach the plasma membrane, as expressing a mutant LRP1 with an ER retention sequence led to a reduction in plasma membrane or Golgi-associated APP and a significant increase in co-localization with mutant LRP1 in the ER. LRP1 deficiency leads to a reduction of cell surface APP and therefore APP internalization and Aβ production[301,303]. *In vivo* experiments using amyloidogenic mouse models crossed with forebrain neuron-specific KO of LRP1 showed increased amyloid burden[304] whereas in other models purported to target hippocampal neurons did not[305], although a GFAP-Cre was used in the latter case, complicating interpretation. At the same time, some have found that LDLR is also able to bind and clear Aβ[306], and LDLR KO in an amyloidogenic AD mouse model did not impact brain amyloid accumulation[161] whereas overexpression of LDLR reduced Aβ levels[162]. Interestingly, the effects of both LDLR and LRP1 on amyloid production, uptake, and clearance appear to be independent of ApoE, however this may be truer for LDLR than for LRP1. Indeed, one hypothesis has been that ApoE4 especially outcompetes Aβ binding to LDLR or LRP1 due to its higher affinity for its receptors, and that because of this, Aβ uptake and clearance is reduced, leading to amyloid accumulation. Evidence for this is still needed, especially for *in vivo* studies, though one study using microdialysis of

Aβ into the mouse brain supports this idea[307]. Recent findings showing relatively similar binding and receptor internalization between ApoE3 and ApoE4 contradict this[190], suggesting more research is needed to better understand the interaction between ApoE, Aβ, and ApoE receptor binding. Nonetheless, it is clear that LRP1 and LDLR play a significant role in the modulation of Aβ production and clearance, and therefore generation of AD pathology, whether ApoE is involved mechanistically or not.

Additional *in vitro* experiments suggest that the fate of uptaken amyloid carried by ApoE by a neuronal cell line seems to also differ based on isoform, with ApoE3 leading to more lysosomal Aβ and Aβ degradation compared to ApoE4[308]. Therefore, it may be the case that altered ApoE dynamics in ApoE4 cells can induce impaired amyloid degradation and thus, accumulation. However, ApoE4 is more prone to getting stuck in endolysosomes and self-aggregate at low pH[169,190]. It is hard to reconcile these data on their own, as it could be expected that since Aβ is purported to bind to ApoE4 more strongly and ApoE4 gets stuck in endolysosomes, there should be more Aβ in the lysosomes and therefore more amyloid being degraded. However, impaired functioning of the endolysosomal system in ApoE4 cells and in AD brains has been extensively characterized[309,310], which is likely a strong contributing factor to ApoE4 cells generating more amyloid and their reduced capacity for clearing the excess Aβ. Indeed, Aβ may in some cases be uptaken and degraded through LC3-associated endocytosis, a non-canonical autophagy pathway where the lipidated, active LC3 protein is recruited to endosomes following uptake of extracellular contents in order to facilitate their degradation in lysosomes[52]. Impairments to this process in microglia increases their inflammatory activation and Aβ levels in the hippocampus of mice[52]. Further, ApoE4 has a higher baseline level of autophagy compared to ApoE3 and impairs autophagic flux[311],

possibly partly due to its effect on lysosomal acidification[193]. Additional support for the role of endolysosomal dysfunction in AD comes from studies assessing genetic risk factors that consistently identify *PICALM* and *BIN1*, both of which encode important proteins involved in clathrin-mediated endocytosis[312]. A recent paper showed that autolysosomal dysfunction precedes Aβ accumulation in perinuclear autophagic vacuoles in neurons[313]. Interestingly, this study identified neurons that have Aβ-positive blebbing autophagic vacuoles surrounding the nucleus that may become the foundation for the majority of plaques generated in an AD mouse model[313].

Altogether, there is a large amount of evidence showing a link between ApoE, cholesterol, and endolysosomal dysfunction with AD. While the role of prenylation in AD pathogenesis and how it is altered by the disease is still being elucidated, some data suggest it plays a critical factor. Because of its impact on ApoE secretion and small GTPase activity, prenylation may be important for some aspects related to the endolysosomal dysfunction and cellular trafficking observed in AD, especially in ApoE4 carriers. It is not known whether prenylation levels are different between ApoE genotypes. Likewise, it is unclear whether prenylation plays a variable role in regulating ApoE secretion based on isoform. It may be inferred that prenylation differentially modulates ApoE dynamics as differences in secretion between isoforms have been observed[66,120,129,130]. This last point is the subject of my research shown in Chapter 2, in which I determine whether geranylgeranylation is important for secretion of ApoE in astrocyte-enriched cultures from ApoE targeted-replacement mice harboring the three human ApoE alleles. I also address whether geranylgeranylation is differentially important for each genotype as it relates to ApoE secretion.

Chapter 2. Regulation of ApoE secretion by the mevalonate pathway

The following work is re-printed (adapted) from: Milstein, JM, Kulas, JK, Kamal, A, Lo, AB, & Ferris, HA. Regulation of Glial ApoE Secretion by the Mevalonate Pathway is Independent of ApoE Isoform. *Journal of Alzheimer's Disease*.

2.1. ABSTRACT

2.1.1. Background

Lipids synthesized in astrocytes are distributed to other brain cells in high-density lipoprotein-like ApoE particles. ApoE, which is a powerful genetic risk factor for developing Alzheimer's disease, is secreted differently depending on genotype. Secretion of ApoE from mouse astrocytes is regulated by the mevalonate pathway.

2.1.2. Objective

We aimed to understand if the regulation of ApoE secretion from astrocytes by the mevalonate pathway was the same between mouse ApoE and ApoE from humanized mice, and if this is impacted by ApoE isoform.

2.1.3. Methods

Astrocyte-enriched glial cultures from wild-type and humanized ApoE targeted-replacement mice were treated with pharmacological inhibitors of various steps along the mevalonate pathway and ApoE in the conditioned media was measured.

2.1.4. Results

We show that statins and prenylation inhibitors, but not specific cholesterol inhibitors, reduce extracellular ApoE lipoparticle levels in astrocyte-enriched glial cultures, and that this occurs in cells harboring either the mouse ApoE or any of the

three human ApoE genotypes to a similar extent. We find that geranylgeranylation modulates ApoE release from astrocytes, and it does so independent of ApoE genotype.

2.1.5. Conclusions

Our results suggest that prenylation broadly regulates ApoE secretion from astrocytes regardless of ApoE genotype, and that this is mediated specifically by geranylgeranylation. Therefore, our data implicates geranylgeranylation as a general mechanism modulating ApoE release from astrocytes, but likely is not responsible for the reported baseline differences in ApoE secretion seen *in vivo* and *in vitro* across genotypes.

2.2. INTRODUCTION

Unlike other organs, the brain must synthesize its own cholesterol, and in the adult brain cholesterol synthesis primarily occurs in astrocytes. Astrocyte-derived cholesterol is then distributed to other cells in apolipoprotein E (ApoE)-containing lipoparticles. There are 3 variants of ApoE in humans, E2, E3, and E4, which differentially impact the risk of developing late-onset Alzheimer's disease (AD), with ApoE4 conferring the greatest risk, ApoE3 inducing moderate risk, and ApoE2 leading to low risk or even protection from AD development[69,83,93,314]. While the differences in AD risk by ApoE genotype are well-established, the underlying mechanism for ApoE-mediated risk remains controversial.

It has been noted that lipids accumulate in the human AD brain, and that iPSC-derived astrocytes harboring ApoE4 have an altered lipidome compared to ApoE3 cells[315]. ApoE lipoparticles in the periphery are known to both deliver cholesterol to and remove cholesterol from cells, in an activity known as reverse cholesterol transport, in an

isoform-specific manner[316]. We have recently demonstrated that astrocyte-derived ApoE particles are also capable of reverse cholesterol transport[317], resulting in decreased amyloid-β (Aβ) production by neurons. Thus, understanding how astrocyte cholesterol and ApoE are regulated and interact has critical implications for understanding the pathogenesis of AD.

Cholesterol is produced by the mevalonate pathway. This pathway has two main arms, the cholesterol biosynthesis arm and the protein prenylation arm. Protein prenylation is a complex post-translational modification by which lipophilic isoprenyl groups are covalently attached to a protein. Specifically, either a 15-carbon farnesyl or a 20-carbon geranylgeranyl group are used for this process, with the former addition being referred to as farnesylation and the latter referred to as geranylgeranylation. Prenylation is imperative for the functioning of a number of proteins, particularly small GTPases in the Rho, Ras, and Rab protein families[236]. The prenylation of these proteins makes them more lipophilic and better able to embed into membranes to carry out their proper functions. Protein prenylation is generally understood to be conducted by three enzymes in mammals, farnesyltransferase (FTase), geranylgeranyltransferase type 1 (GGTase-1), and Rab geranylgeranyltransferase (RabGGTase), although a possible fourth enzyme was identified more recently[236].

Emerging evidence has pointed to a role for protein prenylation in AD and ApoE biology. Compared to age-matched controls, levels of the prenylation substrates farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) are increased in the frontal cortex of males with AD[293–295]. Transcripts of FPP synthase, as well as protein levels of FTase are also elevated in these AD samples[293–295]. This may suggest either an impairment in prenylation that leads to this substrate build-up or a broader alteration in

the mevalonate pathway that then affects substrate production or translation of prenylation enzymes. In the APP/PS1 mouse model of AD, heterozygous deletion of FTase and GGTase-1 reduces Aβ plaque accumulation, and AD mice with FTase haploinsufficiency have attenuated learning and memory impairments[297]. APP/PS1 mice also exhibit increased prenylation compared to WT mice[296], and mice with tauopathy given the FTase inhibitor lonafarnib had lower levels of tau buildup[298]. Altogether, these findings imply a clear connection between prenylation and AD, yet much about this relationship remains to be fully elucidated.

ApoE itself is not prenylated, and while the interplay between prenylation and regulation of ApoE has not been well-studied, one report found that inhibiting geranylgeranylation impairs ApoE secretion from mouse primary mixed glial cultures and mouse hippocampal brain slices[291]. Whether this is a form of regulation for secretion of ApoE lipoparticles from cells harboring human ApoE has not been addressed. Interestingly, the amount of ApoE lipoparticles being secreted changes in an ApoE isoform-dependent manner. ApoE2 lipoparticles are secreted to a higher extent than both E3 and E4, with ApoE4 being secreted the least[66,120,130,318]. This suggests differential regulation of ApoE secretion in the three ApoE isoforms by an as of yet unelucidated mechanism.

Identifying key modulators of ApoE secretion between genotypes may yield novel insights for therapeutic opportunities. Indeed, several lines of experimental evidence suggest that manipulating brain ApoE levels may be beneficial in slowing the onset of AD-associated pathological changes in the brain. ApoE knock-out (KO) mice are protected against the development of parenchymal brain Aβ plaques[319]. Administration of antibodies against ApoE to mouse models of AD reduces brain tau and amyloid

burden[287,320]. Furthermore, recent work has shown that Cre recombinase-mediated genetic disruption of the ApoE gene in astrocytes potently reduces brain pTau burden and dampens neuroinflammation[119]. While targeting ApoE holds great promise for the treatment of AD, loss of peripheral ApoE causes significant dyslipidemia and ApoE KO mice are commonly used as a mouse model of cardiovascular disease[115]. Thus, pharmacologic strategies to selectively change brain ApoE levels or lipidation state are of interest, both as a therapeutic strategy and as a tool to understand ApoE's biological function in brain physiology.

In this study, we sought to determine whether prenylation regulates ApoE secretion in astrocyte-enriched glia cultured from ApoE targeted-replacement (TR) mice harboring human ApoE2, E3, or E4. Furthermore, we aimed to identify whether the impact of prenylation on regulation of ApoE secretion was dependent on ApoE genotype.

2.3. METHODS

2.3.1. Animals

Housing, animal care, and experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the University of Virginia. C57BL6/J, and humanized ApoE2 (strain #:029017), ApoE3 (strain #:029018), and ApoE4 (strain #:027894) mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA).

2.3.2. Astrocyte-enriched primary glial cultures

Astrocyte-enriched glial cultures containing ~90-95% astrocytes and ~5-10% microglia were obtained from postnatal day 3-4 mice using established methods[321]. Cortical tissue was dissected from the brain, meninges were removed, and cortices were

placed in serum-free DMEM (Gibco, 11320-033, Grand Island, NY, USA) on ice. Cortices from 2-3 pups were combined to generate a single N, with each N defined as an individual culture of cells taken from separate animals for independent experiments. The tissue was then mechanically dissociated and digested for 30 minutes at 37°C in a trypsin enzyme solution supplemented with DNAse I (Roche, 10104159001, Basel, Switzerland). Following digestion, cells were centrifuged at 500g for 5 minutes. The supernatant was removed and the cell pellet was resuspended in growth media (DMEM supplemented with 10% FBS and Pen/Strep). The cells were then plated into p75 flasks (Thomas Scientific, 1164G73, Chadds Ford Township, PA, USA) containing growth media. After 7 days, the flasks were shaken at 500rpm on a temperature-controlled orbital shaker at 37°C for a minimum of 4 hours. This shaking method removes most microglia and all OPCs, while astrocyte cells remain adherent to the bottom of the culture flask. Detached microglia and OPCs were aspirated and the remaining astrocytes and microglia were trypsinized and centrifuged at 500g for 5 minutes at room temperature. The cell pellets were then resuspended in growth media and plated in 6- or 12-well plates for experiments. The cells were maintained in this medium until the onset of experiments (7-10 days).

2.3.3. Inhibitors and drug treatments

Simvastatin (Cayman Chemical, 10010344, Ann Arbor, MI, USA), zaragozic acid (MilliporeSigma, Z2626, Burlington, MA, USA), FGTI 2734 (MedChemExpress, HY-128350, Monmouth Junction, NJ, USA), lonafarnib (Cayman Chemical, 11746, Ann Arbor, MI, USA), and cycloheximide (CHX; Sigma-Aldrich, 01810, St. Louis, MO, USA) were each dissolved in DMSO and aliquots were stored at -20°C. Pravastatin (MilliporeSigma, P4498, Burlington, MA, USA) was dissolved in DMSO and stored at -

80°C. GST and RAP-GST were a kind gift made, isolated, and validated by the lab of Dr. Alban Gaultier at University of Virginia[322,323]. Both reagents were stored at -20°C. In all experiments, cells were placed in serum-free DMEM media without antibiotics 24-72 hours prior to inhibitor treatments to allow the cells to equilibrate to serum-free conditions. Cells were then treated with DMSO as a control or with inhibitors in serum-free DMEM media without antibiotics for 24 hours. Afterwards, fresh media with treatments were added to cells and incubated for 72 hours before collection, unless otherwise noted. Throughout the paper, we include the initial 24-hour treatment in our reporting of the length of treatment to minimize confusion of total treatment length.

For experiments assessing ApoE secretion and reuptake, cells were pre-treated with 10μg/mL CHX for 12 hours in serum-free medium. CHX was then removed and the cells were washed three times before the addition of DMSO, 2μM simvastatin, or 10μM FGTI 2734 in the presence of either 200nM GST or 200nM RAP-GST for 6 hours. The cells and conditioned media were then collected for analysis.

2.3.4. Statin rescue experiments

For pravastatin rescue experiments with farnesyl pyrophosphate (FPP; Sigma-Aldrich, F6892, St. Louis, MO, USA), 10μM FPP was added directly to the media along with 10μM pravastatin and incubated for a total of 5 days, as described above. Controls were treated with DMSO and methanol. For simvastatin rescue experiments using FPP, geranylgeranyl pyrophosphate (GGPP; Sigma-Aldrich, G6025, St. Louis, MO, USA), or geranylgeraniol (GGOH; Sigma-Aldrich, G3278, St. Louis, MO, USA), the isoprenoids were pre-incubated with 10% BSA solution for approximately 3 hours at 37°C while shaking. The FPP:BSA, GGPP:BSA, or GGOH:BSA solutions were then diluted to their final concentrations (0.1% BSA, 10μM FPP, 10μM or 20μM GGPP, and 40μM GGOH) in

serum-free media without antibiotics before being added to cells for the lengths of time described above. For these experiments, all conditions were given 0.1% BSA and control samples were treated with appropriate amounts of DMSO and either ethanol (GGOH rescue) or methanol (FPP and GGPP rescues) for each experiment.

2.3.5. Antibodies

All primary and secondary antibodies were diluted in blocking solution containing 5% BSA and 2% horse serum. For experiments using wild-type (WT) murine cells, we used an ApoE antibody from Abcam (ab183597, Cambridge, UK) at a 1:1,000 dilution. We previously validated the specificity of the Abcam ApoE antibody for measuring ApoE in CM[317]. For all experiments using cells from the humanized ApoE TR mice, we used an antibody purchased from Cell Signaling Technologies (13366S, Danvers, MA, USA) that recognizes only human ApoE, diluted 1:500. The FDFT1 antibody was purchased from Abcam (ab195046, Cambridge, UK) and diluted 1:1,000. The ApoJ alpha chain antibody was purchased from Abcam (ab184100, Cambridge, UK) and used at a 1:1,000 dilution. The HDJ2 antibody was purchased from Fisher Scientific (PIMA5-12748, Waltham, MA, USA) and used at a 1:1,000 dilution. The RhoA antibody was purchased from Cell Signaling (2117S, Danvers, MA, USA) and diluted 1:250. The IRβ antibody was purchased from EMD Millipore (MABS65, Burlington, MA, USA) and used at a 1:500 dilution.

The corresponding secondary antibodies for western blotting were diluted 1:2,500 in the same 5% BSA and 2% horse serum blocking solution. Secondary antibodies included donkey anti-rabbit AlexaFluor 647 Plus (Invitrogen, A32795, Carlsbad, CA, USA) and goat anti-mouse DyLight800 (Bio-Rad, STAR117D800GA, Hercules, CA, USA). Rhodamine-conjugated actin antibody (BioRad, 12004163,

Hercules, CA, USA) was used as a loading control for cell lysate experiments at a dilution of 1:5,000.

2.3.6. Sample collection and processing

Following drug treatment, conditioned media (CM) was collected and cleared of cellular debris by centrifugation at 10,000g for 3 minutes at room temperature. 500uL of each CM sample was then concentrated using Microcon 10kDa filter membranes (MilliporeSigma, MRCPRT010, Burlington, MA, USA) by centrifugation at 14,000g for 20 minutes at room temperature. 4x Laemmli SDS sample buffer supplemented with 2-mercaptoethanol was then added to the concentrated CM. The samples were denatured by heating at 90°C for 10 minutes.

In measurements of cell lysate proteins, cells were lysed on ice in either PBS or RIPA buffer (bioWORLD, 42020024, Dublin, OH, USA) containing protease and phosphatase inhibitors. The samples were then sonicated for 10 seconds and cell lysate protein was quantified by BCA assay (Thermo Scientific, 23227, Waltham, MA, USA). The samples were diluted in Laemmli SDS sample buffer (Bio-Rad, 1610737, Hercules, CA, USA), and boiled at 90°C for 10 minutes.

2.3.7. Cell membrane and cytosol fractionation

Cellular fractionation was conducted with a modified protocol using the extraction buffers (supplemented with protease inhibitor cocktail and DTT) from the Abcam Fraction-PREP Cell Fractionation Kit (Abcam, ab288085, Cambridge, UK). Following treatment, cell lysate was collected in ice-cold PBS with protease and phosphatase inhibitor cocktails. To wash the samples, the lysate was pelleted at 750g for 5 minutes at 4°C, supernatant was discarded, and the pellet was resuspended in ice-cold PBS. The lysate was again spun at 750g for 5 minutes at 4°C and supernatant was discarded. Cell

lysate was resuspended in 100µL of Cytosol Extraction Buffer from the Abcam fractionation kit. The samples then underwent three freeze-thaw cycles, and were subsequently placed on a rotator for 20 minutes at 4°C. Samples were centrifuged at 1,250g for 10 minutes at 4°C, and the resulting supernatant (cytoplasmic fraction) was collected and processed for western blot. The pellet from this step was washed twice with PBS, with spins at 1,250g for 5 minutes each at 4°C. The washed pellet was then resuspended in 100µL of Membrane Extraction Buffer-A (MEB-A) and vortexed for 20 seconds at 5 second intervals. Membrane Extraction Buffer-B (MEB-B) was then added and the samples were vortexed again for 20 seconds at 5 second intervals. Samples were incubated on ice for approximately 2 minutes before being vortexed for another 20 seconds at 5 second intervals and centrifuged at 1,500g for 5 minutes at 4°C. The supernatant (membrane fraction) was collected and processed for western blot. For these samples, neither the cytosolic nor membrane fractions were boiled to avoid potential membrane protein aggregation, but both fractions were sonicated for 10 seconds.

2.3.8. SDS-PAGE and western blot

SDS-PAGE was performed using Bio-Rad 4-20% Stain-Free polyacrylamide gels (Bio-Rad, 4568096 or 4568094, Hercules, CA, USA). A molecular weight marker was used in all experiments (Bio-Rad, 1610373, Hercules, CA, USA). Each experiment using cell lysate was run with equal concentrations of protein (at least 10µg/well), while CM was compared by running the same volume of CM from each well, concentrated in parallel. The gels were then transferred to low-fluorescence PVDF membranes (Bio-Rad, 1704274, Hercules, CA, USA) using a Bio-Rad Trans-Blot Turbo. Membranes were blocked for at least 2 hours using blocking solution. Primary antibodies were diluted in

blocking solution as described above and applied to membranes overnight at 4°C or at room temperature on a rocker. After washing with PBST solution, fluorescent secondary antibodies were incubated on membranes for at least 2 hours at room temperature. Fluorescent signal was imaged with a Bio-Rad ChemiDoc MP imaging system (Bio-Rad, Hercules, CA, USA).

2.3.9. ApoE ELISA

Extracellular ApoE levels were assessed using either a human ApoE ELISA kit (Abcam, ab108813, Cambridge, UK) or a mouse ApoE ELISA kit (Abcam, ab215086, Cambridge, UK) according to manufacturer's instructions. Unconcentrated CM from ApoE TR cells was diluted between 1:20 and 1:50 in sample diluent. For mouse ApoE ELISAs, unconcentrated CM from WT cells was diluted between 1:2 and 1:200 in sample diluent. Each biological sample was run in technical duplicates. Absorbance was measured at 450nm with a background subtraction reading at 570nm for the human ApoE ELISAs. For normalization, total cell lysate protein was measured by BCA assay (Thermo Scientific, 23227, Rockford, IL, USA). ApoE levels are expressed as nanogram or milligram of secreted ApoE per milligram of total cell lysate protein.

2.3.10. qPCR

For qPCR assessment of mevalonate pathway and cholesterol-responsive gene transcription, mRNA was isolated using the Bioline ISOLATE II RNA Mini Kit (BIO-52073, London, England). Between 100ng and 1µg of mRNA was used for cDNA synthesis with iScript Reverse Transcription Supermix (Bio-Rad, 1708841, Hercules, CA, USA). Transcripts were measured by Sybr Green qPCR assay on a Bio-Rad CFX384 qPCR machine. Cq values were calculated and normalized to *Tbp* expression. Primers sequences used were as follows: *Hmgcr* (FW: TCAGTGGGAACTATTGCACCGACA,

RV: TGGAATGACGGCTTCACAAACCAC), *Srebf2* (FW: GCGTTCTGGAGACCATGG, RV: ACAAAGTTGCTCTGAAAACAAATC), *Fdft1* (FW: AGCTCACCTGAAAGCCCAGAAAGA, RV: TGCCTGCTTTCCTTACCCTCATCA), *Lxrβ* (FW: GTTGCTTCGAGCTACTCCCA, RV: GCGAGAGTTGCCTCTGTGTC), *Tbp* (FW: ACCCTTCACCAATGACTCCTATG, RV: TGACTGCAGCAAATCGCTTGG).

2.3.11. Cell viability assays

CCK-8 assays (Dojindo, CK04, Munich, Germany) to assess cell number and therefore viability were performed by following the manufacturer's instructions. Briefly, cultures were grown in 96-well clear sterile assay plates. Following drug treatment, 10μL of CCK-8 solution was added to each well. The plate was incubated for 1-2 hours and absorbance was measured at 450nm by plate reader. The absorbance is proportional to the number of viable cells in each well. Data is expressed as a percentage compared to the number of viable cells in control samples.

2.3.12. Statistical analysis

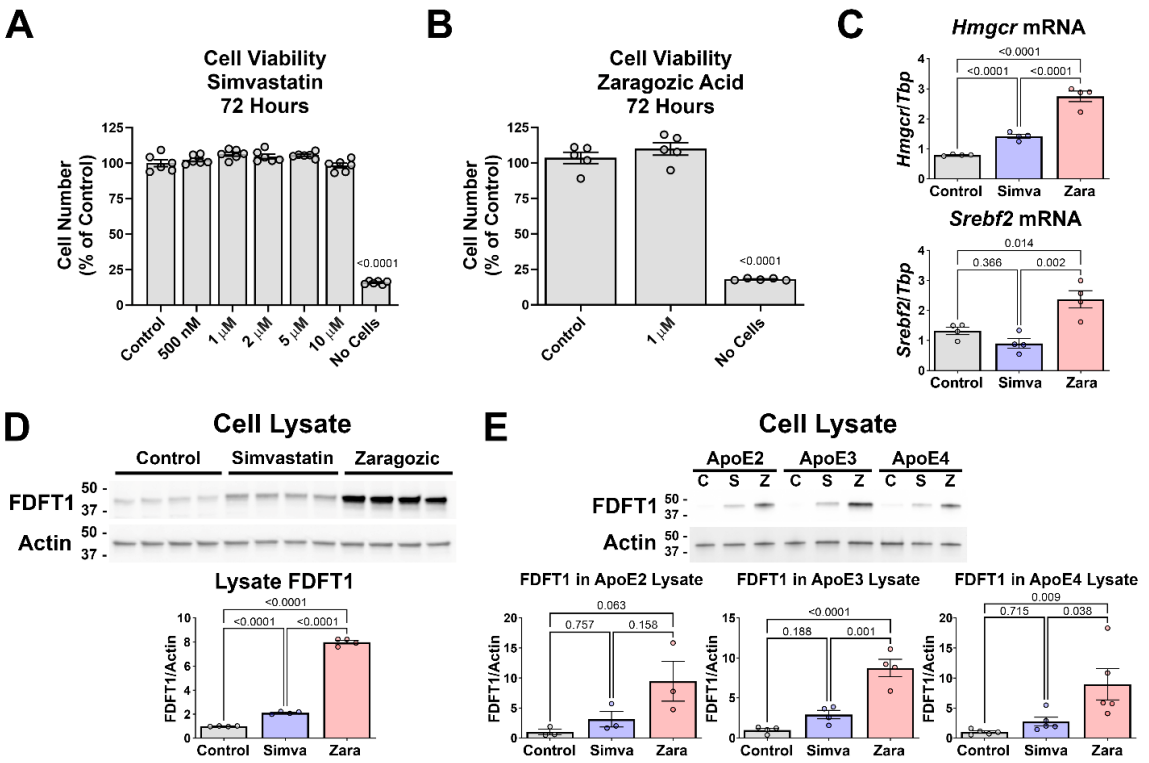
Statistical analysis and graphing of data was performed using GraphPad Prism 10 software (GraphPad Software, La Jolla, CA, USA). Tukey's post-hoc testing for multiple comparisons was used in the case of one-way and two-way ANOVA.

2.4. RESULTS

2.4.1. Statins, but not cholesterol synthesis inhibitors, impair ApoE secretion in ApoE TR glia

In our recent work, we observed that cultured mouse astrocytes secrete large amounts of ApoE protein into culture media, which can be reliably measured by western blot[317]. Based on previous findings from our lab showing a strong effect of ApoE on Aβ

generation depending on the lipidation status of the particle[317] and data from others finding statins reduce ApoE secretion from glia[291], we hypothesized that blocking the mevalonate pathway both upstream with statins and downstream with cholesterol biosynthesis inhibitors would impact ApoE secretion. To test this hypothesis, we first utilized two inhibitors along the mevalonate pathway, the HMGCR inhibitor simvastatin and the FDFT1 inhibitor zaragozic acid. The drugs were applied to either wild-type (WT) murine glia or glia from ApoE TR mice for 24 hours and then the media and drugs were replaced. This refreshed media was conditioned on the cells for 72 hours before sample collection (referred to hereafter as 96 hours of treatment; Figure 2.1A-B). We first assessed the toxicity of increasing doses of simvastatin and 1μM zaragozic acid placed on WT glia for 72 hours and found no significant toxic effects (Supplementary Figure 2.1A-B). To validate that our drug treatments worked as expected, we measured transcript levels of *Hmgcr* and *Srebf2* in WT cells treated with simvastatin (2μM) or zaragozic acid (1μM) and observed an increase in *Hmgcr* transcripts for both treatments, as well as an increase in *Srebf2* transcripts in zaragozic acid-treated cells (Supplementary Figure 2.1C). To further validate our drug treatments, we measured cellular protein levels of FDFT1 by western blot (WB) and saw an increase in the amount of FDFT1 in both treatment groups compared to controls in both WT and ApoE TR glia (Supplementary Figure 2.1D-E). Together, these data suggest that both simvastatin and zaragozic acid are acting on their targets as expected, as treatment led to compensatory upregulation of genes and proteins in the mevalonate pathway.



Supplementary Figure 2.1. Inhibition of the mevalonate pathway by simvastatin and zaragozic acid. (A) Wild-type (WT) astrocyte-enriched cultures were treated with different doses of simvastatin for 72 hours and cell number and therefore cell viability was assessed by CCK-8 assay. Data is expressed as a percentage of living cells compared to controls. N=6 biological replicates/condition, one-way ANOVA. **(B)** WT astrocyte-enriched cultures were given DMSO as a control or 1μM zaragozic acid treatment for 72 hours and cell viability was measured by CCK-8 assay. N=5 biological replicates/condition, one-way ANOVA. **(C)** Astrocyte-enriched glia from WT mice were treated with 2μM simvastatin or 1μM zaragozic acid for 96 hours and transcripts for the mevalonate pathway enzyme genes Hmgcr and Srebf2 were measured by qPCR. N=4 biological replicates/condition, one-way ANOVA. **(D)** WT cells were treated with 2μM simvastatin or 1μM zaragozic acid for 96 hours and cell lysate was analyzed for FDFT1 levels to confirm drug efficacy. N=4 biological replicates/condition, one-way ANOVA. **(E)** Astrocyte-enriched cultures from ApoE TR mice were treated with 2μM simvastatin or 1μM zaragozic acid for 96 hours and FDFT1 levels were measured to show drug targeting efficiency. N=3-5 biological replicates/condition/genotype, one-way ANOVA. All data are normalized to controls and graphed as mean ± s.e.m. C, control; S or Simva, simvastatin; Z or Zara, zaragozic acid.

To address the hypothesis that cholesterol synthesis modulates ApoE lipoparticle secretion, we collected equal volumes of conditioned media (CM) and measured extracellular ApoE levels in concentrated CM. We observed that while simvastatin and zaragozic acid had no significant impact on intracellular ApoE in WT cells (Figure 2.1C), simvastatin significantly reduced CM ApoE levels in a dose-dependent manner, whereas no significant effect was observed on ApoE in CM from zaragozic acid-treated cells (Figure 2.1D). This suggested that inhibition of an arm of the mevalonate pathway other than cholesterol synthesis is important for lipoprotein release from cells. Of note, the 5μM and 10μM doses of simvastatin showed clear changes in the total protein signature in the CM, suggesting the drugs were beginning to have toxic effects at these higher concentrations after 96 hours of treatment, although CCK-8 cell viability assays showed no significant cytotoxicity after 72 hours of treatment (Supplementary Figure 2.1A).

The sequence homology between mouse and human ApoE is only about 70%[324], and the ApoE promoter has just a 40% sequence homology between the two species[325]. Aside from this, there are known differences between mouse and human ApoE, including how they impact AD pathology in mouse models[326]. Importantly, unlike mice, humans have three ApoE isoforms, E2, E3, and E4, which have highly variable biochemical properties. Of the human isoforms, ApoE2 lipoparticles are both lipidated and secreted from cells to the highest extent, followed by ApoE3 and ApoE4, respectively[66,130,318].

Due to these reported differences between mouse and human ApoE, we tested the effects of simvastatin and zaragozic acid in cells harboring human ApoE2, ApoE3, or ApoE4 to determine whether statins regulate ApoE secretion to a similar extent in ApoE TR cells, and if they do so differentially based on ApoE genotype. Treating cells with

2μM simvastatin for 96 hours led to a 36-50% reduction in extracellular ApoE secreted from astrocytes no matter the genotype, while intracellular ApoE levels remained unaffected (Figure 2.1E-F). Similar to WT cells, 1μM zaragozic acid treatment did not change intracellular ApoE levels in any genotype, although ApoE levels within ApoE4 cells had a trend towards an increase after zaragozic acid treatment that did not reach statistical significance (Figure 2.1E). Nonetheless, regardless of ApoE genotype, cholesterol synthesis inhibition did not lead to changes in ApoE secretion (Figure 2.1F). Thus, our data show that statins impair secretion of ApoE from astrocytes for all ApoE isoforms to a similar extent, and that inhibition of cholesterol synthesis alone is not sufficient to explain this effect.

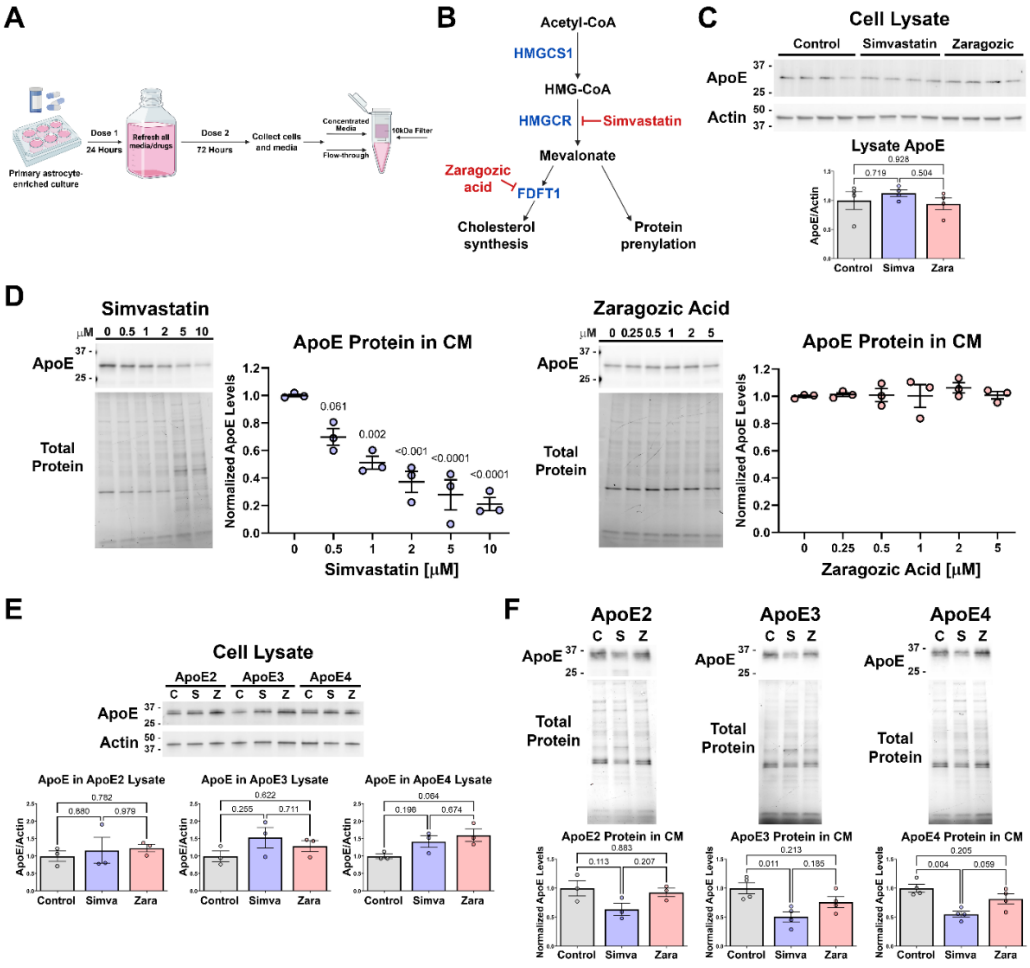
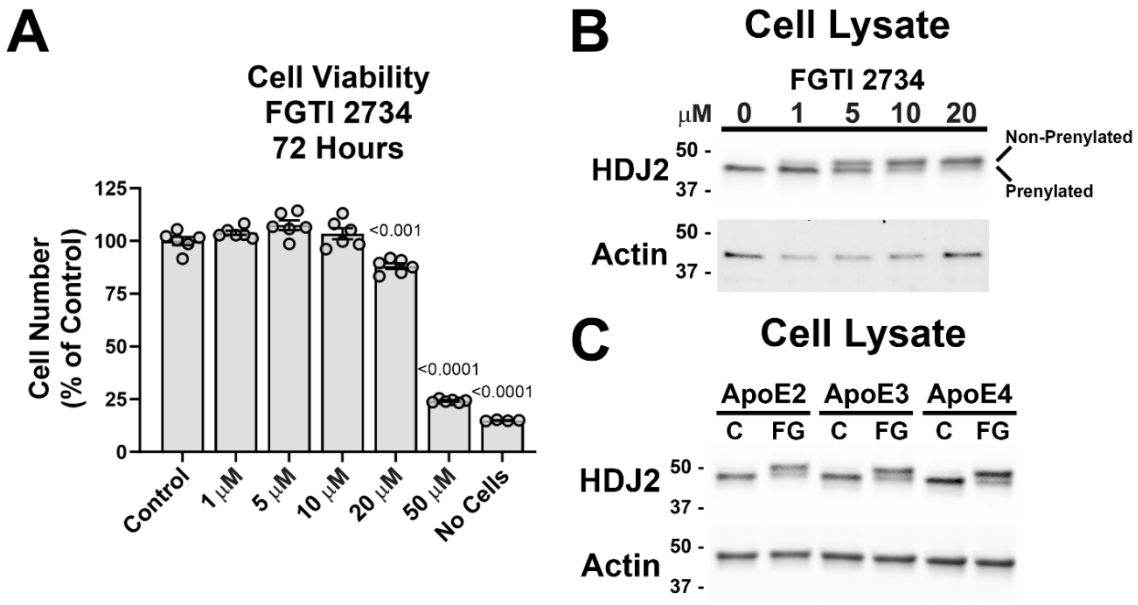


Figure 2.1. Statins, but not cholesterol synthesis inhibitors, impair ApoE secretion in ApoE TR glia. (A) Diagram depicting the treatment protocol used for the current study. **(B)** Simplified diagram of the mevalonate pathway, showing its two primary arms, the cholesterol synthesis arm and protein prenylation arm. Points of the pathway at which the drugs simvastatin and zaragozic acid inhibit the pathway are shown in red. Key enzymes in the pathway are shown in blue. **(C)** Astrocyte-enriched primary glial cultures were treated with DMSO, 2μM simvastatin, or 1μM zaragozic acid for a total of 96 hours. Cellular ApoE levels were measured and their relative abundance compared to actin was quantified. N=4 biological replicates/condition, one-way ANOVA. **(D)** Cells were treated with increasing concentrations of simvastatin or zaragozic acid for a total of 96 hours. Conditioned media (CM) was concentrated and extracellular ApoE protein levels were measured by western blot. N=3 biological replicates, one-way ANOVA. **(E)** Astrocyte-enriched glial cultures from ApoE targeted-replacement (TR) mice were treated with DMSO, 2μM simvastatin, or 1μM zaragozic acid for a total of 96 hours. Cellular ApoE levels were measured and their relative abundance compared to actin was quantified. N=3 biological replicates/condition/genotype, one-way ANOVA. **(F)** Astrocyte-enriched glia from ApoE TR mice were treated with 2μM simvastatin, or 1μM zaragozic acid for a total of 96 hours and extracellular CM ApoE levels were measured. N=3-4 biological replicates/condition/genotype, one-way ANOVA. All data are normalized to controls and graphed as mean ± s.e.m. C, control; CM, conditioned media; S or Simva, simvastatin; Z or Zara, zaragozic acid.

2.4.2. ApoE secretion from astrocytes is regulated by protein prenylation

The mevalonate pathway has two main arms, the cholesterol biosynthesis arm and the protein prenylation arm (Figure 2.1B). As inhibiting both arms of the pathway with simvastatin impaired ApoE secretion, but inhibiting just the cholesterol biosynthesis arm with zaragozic acid did not, we sought to focus on the role of protein prenylation on glial ApoE secretion. While ApoE itself is not prenylated, one paper has suggested that prenylation mediates proper ApoE secretion from murine mixed glial cultures and cultured brain slices[291]. Therefore, we first aimed to confirm the findings of this paper using different and more specific inhibitors of protein prenylation.

To confirm that statins impair protein prenylation in our cultures, we used the farnesylated protein HDJ2 as a proxy readout for measuring prenylation inhibition due to a difference in its gel mobility between its prenylated and non-prenylated forms[327]. Indeed, treatment of astrocyte-enriched glial cultures with simvastatin led to a decrease in the prenylated-to-non-prenylated HDJ2 ratio, confirming impaired prenylation as a result of statin treatment (Figure 2.2A).



Supplementary Figure 2.2. FGTI 2734 inhibits cellular protein prenylation. (A) WT cells were treated with increasing doses of the dual FTase and GGTase-1 inhibitor FGTI 2734 for 72 hours. Cell numbers were measured by CCK-8 cell viability assays to determine pharmacological cytotoxicity. N=4-6 biological replicates/condition, one-way ANOVA. **(B)** Astrocyte-enriched glia from WT mice were treated with increasing concentrations of FGTI 2734 and cell lysate was collected and run on western blots to assess drug efficacy by observing gel mobility shift for the farnesylated protein HDJ2. **(C)** ApoE TR astrocyte-enriched glia were treated with 10μM FGTI 2734 for 96 hours and HDJ2 mobility shift was assessed in cell lysates to show drug-induced prenylation inhibition. Data are normalized to control and presented as mean + s.e.m. C, control; FG, FGTI 2734.

To confirm that the prenylation arm of the mevalonate pathway specifically plays a role in regulating ApoE release, we utilized a recently-developed and characterized dual inhibitor of FTase and GGTase-1 called FGTI 2734 (Figure 2.2B)[327]. Astrocyte-enriched glial cultures from WT mice were treated with increasing doses of FGTI 2734 for 96 hours and cell lysate and CM were collected for analysis. Cell viability assessment using CCK-8 assays revealed cytotoxicity of the drug only at very high concentrations after 72 hours of treatment (Supplementary Figure 2.2A). We observed a clear shift in the ratio of prenylated to non-prenylated HDJ2 after treatment, with increasingly higher doses of FGTI 2734 resulting in higher amounts of non-prenylated HDJ2, demonstrating the effectiveness of the drug as a prenylation inhibitor (Supplementary Figure 2.2B). We then treated astrocyte-enriched glia with the same range of FGTI 2734 concentrations in order to assess its impact on ApoE secretion. Similar to our findings using simvastatin, FGTI 2734 reduced extracellular ApoE levels in a dose-dependent manner (Figure 2.2C). Interestingly, FGTI 2734 treatment did not impact extracellular levels of another secreted protein, ApoJ (Figure 2.2C), which may reflect some specificity in the secretory pathways being impacted by FGTI 2734 treatment.

We then treated ApoE2, E3, and E4 glial cultures with 10μM FGTI 2734 for 96 hours and found that in all genotypes, drug treatment led to impaired HDJ2 prenylation (Supplementary Figure 2.2C) and ApoE secretion as measured by extracellular ApoE content (Figure 2.2D). ApoE amounts in the conditioned media were reduced to similar levels (~40-51%) for all three ApoE isoforms in response to FGTI 2734 treatment. This suggests that prenylation by FTase and/or GGTase-1 has a large impact on regulating the pool of secreted ApoE lipoparticles irrespective of ApoE genotype.

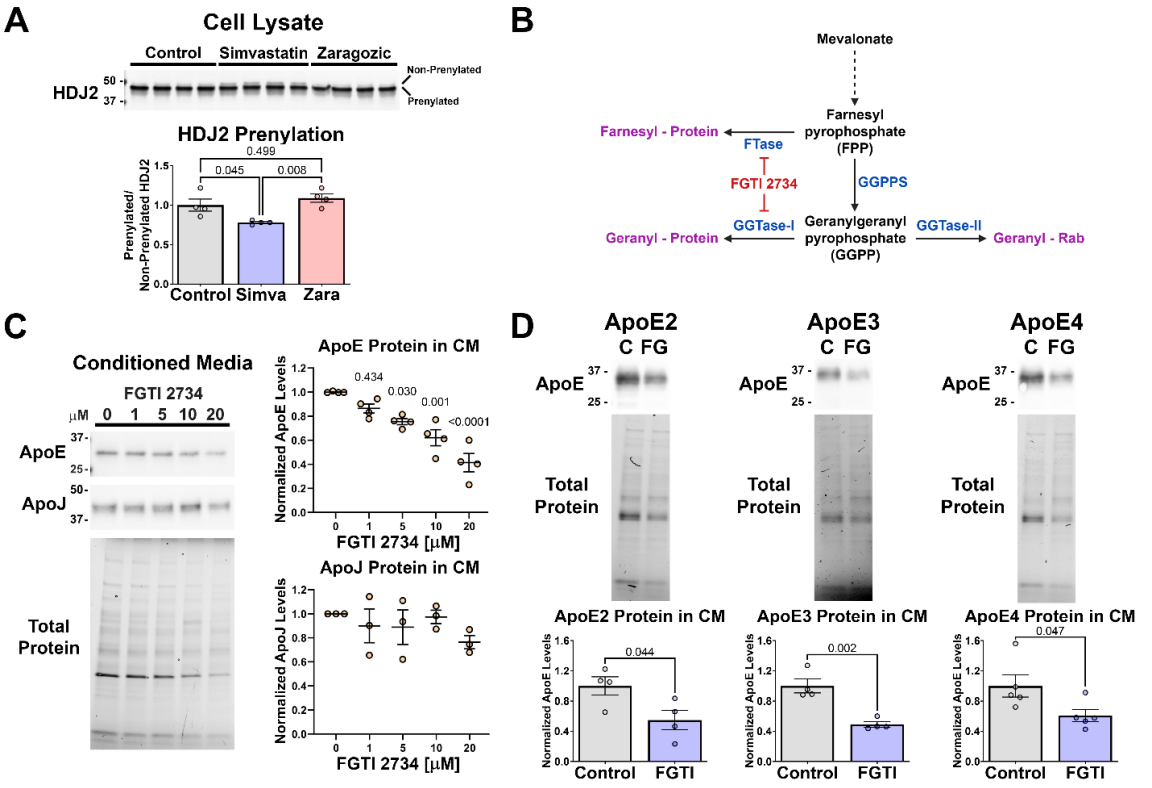
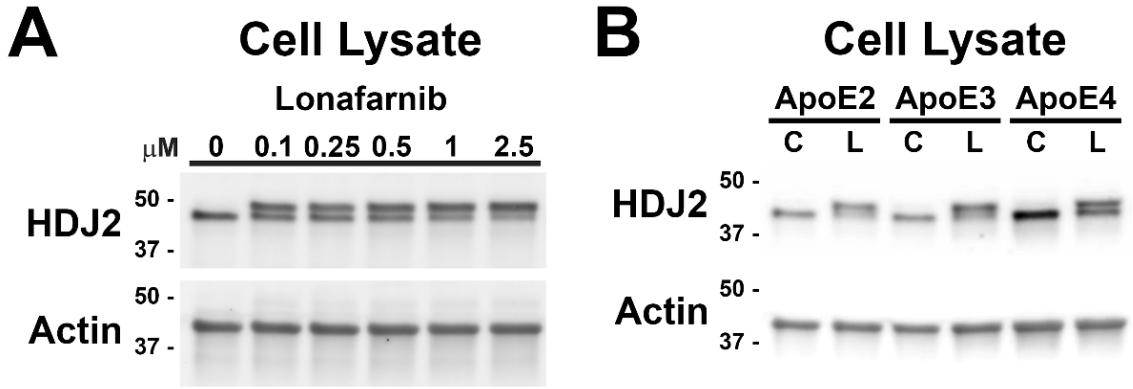


Figure 2.2. Prenylation modulates secretion of ApoE lipoparticles from glia. (A) WT astrocyte-enriched glial cultures were treated with 2μM simvastatin, or 1μM zaragozic acid for 96 hours and cell lysate was collected in order to measure mobility shift of the farnesylated protein HDJ2 in response to treatment. The prenylated to non-prenylated HDJ2 ratio was quantified. N=4 biological replicates/condition, one-way ANOVA. **(B)** Diagram showing the prenylation arm of the mevalonate pathway, as well as the prenylation inhibitor FGTI 2734 used for experiments shown in red. Enzymes are shown in blue, with prenylation products shown in purple. **(C)** Conditioned media (CM) from WT cells treated with increasing doses of FGTI 2734 was visualized by western blot to measure relative extracellular ApoE and ApoJ protein levels. N=3-4 biological replicates/condition, one-way ANOVA. **(D)** Astrocyte-enriched glial cultures from ApoE TR mice were treated with 10μM FGTI 2734 for 96 hours. Secreted ApoE levels in concentrated conditioned media was assessed by western blot. N=4-5 biological replicates/condition/genotype, Student's t-test. All data are normalized to controls and expressed as mean ± s.e.m. C, control; CM, conditioned media; FG or FGTI, FGTI 2734; Simva, simvastatin; Zara, zaragozic acid.

2.4.3. Farnesylation alone does not impact ApoE lipoparticle secretion

As FGTI 2734 impacts the activity of both FTase and GGTase-1, this leaves open the potential for either farnesylation or geranylgeranylation to be critical for proper ApoE secretion. Additionally, it may be the case that alternatively prenylated proteins mediate this process. Therefore, to better understand which type of prenylation is the primary mediator of the effects of FGTI 2734 on ApoE, we used a highly specific and potent inhibitor of FTase called lonafarnib (Figure 2.3A).



Supplementary Figure 2.3. Lonafarnib inhibits farnesylation of proteins. (A) Astrocyte-enriched cultures from WT mice were treated with increasing amounts of the FTase inhibitor lonafarnib for 96 hours. HDJ2 mobility shift was observed to show inhibition of protein farnesylation by drug treatment. **(B)** Astrocyte-enriched glial cultures harboring different ApoE alleles were treated with 500nM lonafarnib for 96 hours and HDJ2 mobility shift was assessed to measure drug efficacy between genotypes. C, control; L, lonafarnib.

When we treated WT astrocyte-enriched glial cultures with increasing doses of lonafarnib, we could see a clear shift in HDJ2 to its non-prenylated form, confirming the drug worked as expected (Supplementary Figure 2.3A). As predicted based on previous findings[291], we did not observe any effect of lonafarnib on the amount of extracellular ApoE at any of the tested doses (Figure 2.3B), indicating that farnesylation alone does not mediate ApoE release from murine astrocytes.

In cultures from ApoE TR mice, we found that treatment with 500nM lonafarnib for 96 hours impaired HDJ2 prenylation in all genotypes, suggesting no difference in drug efficacy based on ApoE genotype (Supplementary Figure 2.3B). Similarly, FTase inhibition alone did not modulate extracellular ApoE levels from cells of any of the human ApoE isoforms (Figure 2.3C). Altogether, our data show clearly that farnesylation alone does not mediate secretion of ApoE lipoparticles for any ApoE isoform.

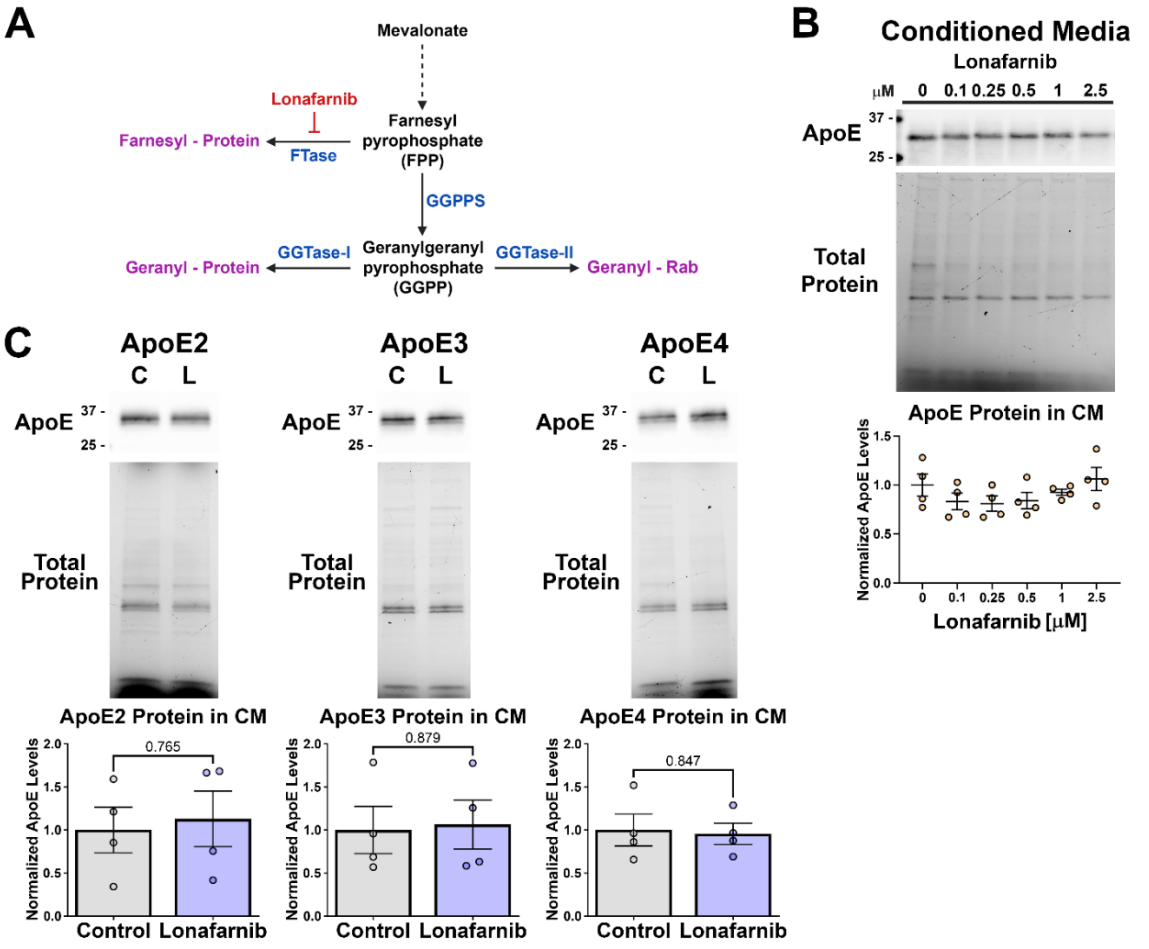


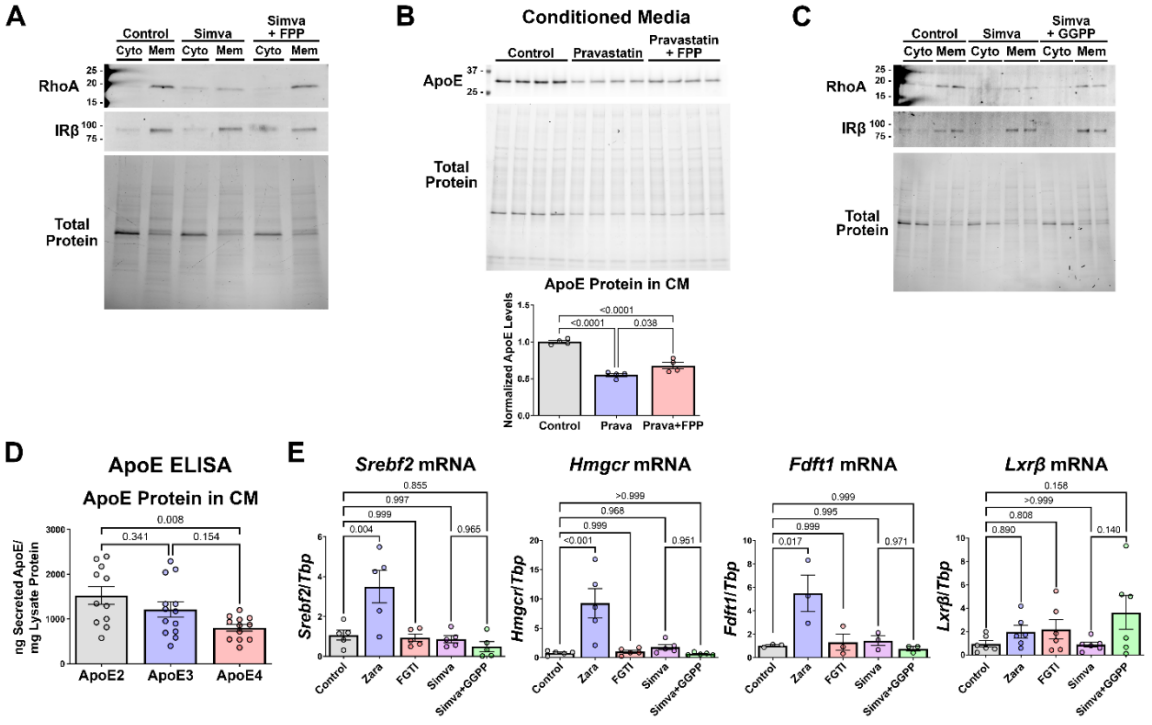
Figure 2.3. Farnesylation alone does not mediate proper secretion of ApoE. (A) Diagram showing the prenylation arm of the mevalonate pathway, as well as the FTase inhibitor lonafarnib used for experiments shown in red. **(B)** WT astrocyte-enriched glia were treated with increasing doses of lonafarnib for 96 hours and conditioned media (CM) was assessed for ApoE. N=4 biological replicates/condition, one-way ANOVA. **(C)** Secretion of ApoE was measured by western blot from astrocyte-enriched glial cultures harboring the three human ApoE genotypes after 96 hours of 500nM lonafarnib treatment. N=4 biological replicates/condition, Student's t-test. All data are normalized to controls and presented as mean + s.e.m. C, control; CM, conditioned media; L, lonafarnib.

2.4.4. Geranylgeranylation-mediated ApoE secretion occurs independent of ApoE genotype

Prenylation involves the post-translational enzymatic addition of isoprenoids to proteins. There are three key enzymes widely accepted to prenylate proteins in mammalian systems[236]. FTase utilizes the 15-carbon isoprenoid FPP as a substrate to add to a variety of proteins. FPP can then be catabolized into the 20-carbon isoprenoid GGPP, which in turn can be used by both GGTase-1 and RabGGTase (Figure 2.2B).

We sought to confirm whether geranylgeranylation alone is sufficient for regulating secretion of ApoE from astrocyte-enriched glial cultures, and whether this is the case in the three human ApoE genotypes. To do so, we performed rescue experiments using different prenylation substrates to show the importance of geranylgeranylation. First, we treated WT cells with simvastatin in the presence or absence of FPP and found that simvastatin treatment reduced membrane association of RhoA and that FPP add-back rescued this effect, suggesting the FPP rescue worked (Supplementary Figure 2.4A). FPP supplementation in the presence of simvastatin also rescued the reduction of extracellular ApoE levels (Figure 2.4A). To further validate our simvastatin effect and to show the impact of adding back a prenylation substrate, we

treated cells with pravastatin, a less potent, more hydrophilic statin compared to simvastatin, with or without FPP. Our pilot experiments suggested pravastatin is well-tolerated by astrocytes, though its effects are milder. We treated WT cultures for a total of 5 days with 10μM pravastatin and observed a strong reduction in CM ApoE protein (Supplementary Figure 2.4B). Adding back the prenylation substrate FPP in combination with pravastatin yielded a moderate, but significant, rescue of the pravastatin-induced reduction in extracellular ApoE (Supplementary Figure 2.4B), confirming the importance of prenylation for proper secretion of ApoE.



Supplementary Figure 2.4. GGPP rescue of simvastatin-induced impairment of RhoA geranylgeranylation. (A) Astrocyte-enriched glial cultures from WT mice were treated with 2μM simvastatin with or without supplementation of 10μM farnesyl pyrophosphate (FPP) for 96 hours. To show efficacy of FPP add-back, cell lysate was fractionated and both cytosolic (Cyto) and membrane (Mem) fractions were run on a WB for membrane-bound RhoA. The membrane-associated insulin receptor β subunit (IRβ) was used to show fractionation efficiency. N=1 biological replicate/condition. B) Astrocyte-enriched glial cultures from WT mice were treated with 10μM pravastatin for 5 days with or without 10μM (FPP). Conditioned media (CM) was concentrated and run on a WB to measure extracellular ApoE levels. N=4 biological replicates/condition, one-way ANOVA. C) Astrocyte-enriched cultures from WT mice were treated with either 2μM simvastatin or simvastatin with 20μM GGPP for 96 hours. Membrane fractionation of cell lysate was performed and cytosolic and membrane fractions were run on a WB to assess membrane association of RhoA. N=2 biological replicates/condition. D) Unconcentrated conditioned media (CM) levels of extracellular ApoE from ApoE TR astrocyte-enriched cultures at baseline was assessed by ELISA. N=11 biological replicates/genotype, one-way ANOVA. E) Astrocyte-enriched glial cultures from ApoE3 TR mice were treated with either DMSO as a control, 1μM zaragozic acid, 10μM FGTI 2734, 2μM simvastatin, or 2μM simvastatin with 20μM GGPP for 96 hours. qPCRs for cholesterol-responsive gene transcripts were conducted following mRNA extraction. N=3-6 biological replicates/condition, one-way ANOVA. All data are presented as mean ± s.e.m. CM, conditioned media; Cyto, cytosolic fraction; FGTI, FGTI 2734; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; IRβ, insulin receptor β subunit; Mem, membrane fraction; Prava, pravastatin; Simva, simvastatin; Zara, zaragozic acid.

To get a more direct confirmation of the role of geranylgeranylation in mediating ApoE secretion, we used the GGTase-1 substrate GGPP and its alcohol form GGOH to rescue the effects of simvastatin treatment. When we treated WT cells with simvastatin supplemented with either GGPP or GGOH, we were able to observe a significant rescue, where ApoE levels in CM matched those of untreated cells (Figure 2.4B). To confirm that our rescue worked as intended, we performed cell fractionation assays and measured membrane-associated RhoA in control-, simvastatin-, and simvastatin with

GGPP-treated cells. We found less membrane-associated RhoA in simvastatin-treated cells compared to controls, and a rescue of this deficit by GGPP addition (Supplementary Figure 2.4C). These data validate our GGPP rescue experiment and strengthens our finding that geranylgeranylation modulates ApoE secretion from WT astrocytes.

We next sought to determine whether geranylgeranylation plays a role in regulating ApoE release from cells harboring human ApoE2, E3, or E4, and if so, whether the extent to which geranylgeranylation is involved in secretion of ApoE lipoparticles differs between isoform. We first quantified extracellular ApoE content secreted from ApoE TR glia and were able to confirm that ApoE2 astrocytes release more ApoE lipoparticles compared to ApoE4 astrocytes at baseline using ELISAs (Supplementary Figure 2.4D). We found that giving back GGPP or GGOH in simvastatin-treated cells yielded a statistically significant rescue of extracellular ApoE levels in glia from all ApoE genotypes (Figure 2.4C).

It may be the case that inhibition of prenylation or adding back prenylation substrates modulates cholesterol-responsive elements that may in turn impact ApoE levels. To test this, we treated astrocyte-enriched glial cultures from ApoE3 TR mice with DMSO as a control, 1μM zaragozic acid, 10μM FGTI 2734, 2μM simvastatin, simvastatin with 20μM GGPP supplementation for 96 hours and assessed transcripts of genes involved in cholesterol synthesis and sensing. We found that only zaragozic acid significantly increased transcripts of *Srebf2*, *Hmgcr*, and *Fdft1*, while no treatment impacted *Lxrβ* transcripts (Supplementary Figure 2.4E). Therefore, we believe that prenylation inhibition and GGPP add-back do not induce cholesterol-responsive

elements and that these factors do not underlie our observed effects on extracellular ApoE content.

Altogether our data from astrocyte-enriched glial cultures from ApoE TR mice show that inhibition of the prenylation pathway by statins impairs ApoE secretion regardless of ApoE isoform, and suggests that geranylgeranylation specifically modulates extracellular ApoE lipoparticle levels independent of ApoE genotype.

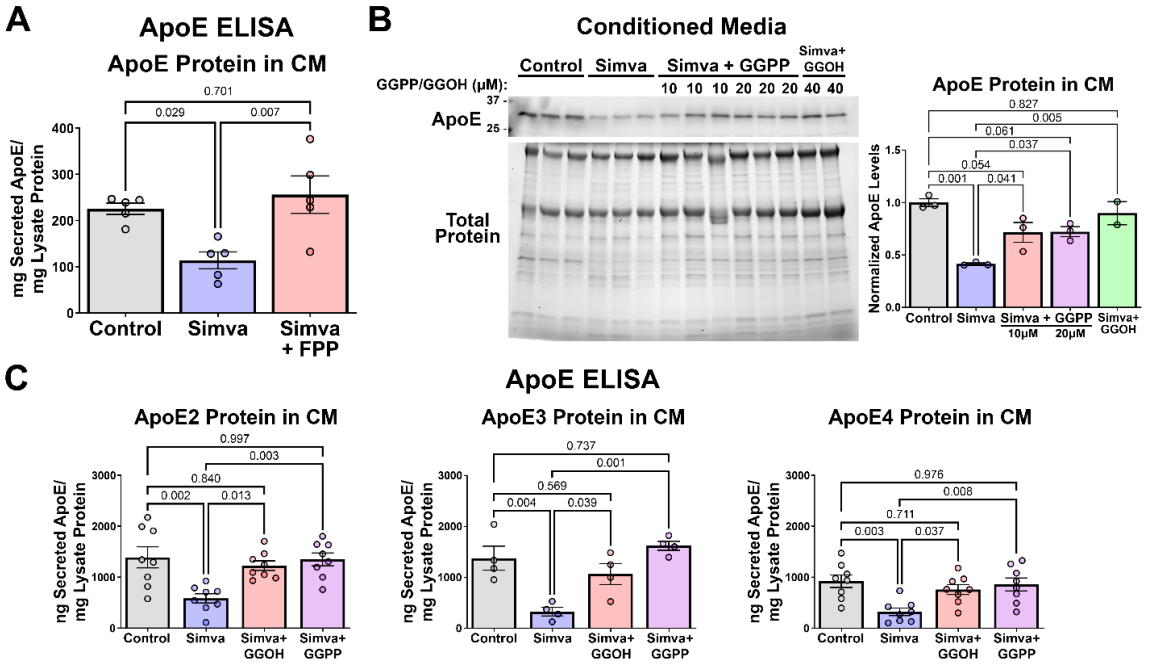


Figure 2.4. Geranylgeranylation regulates ApoE secretion independent of ApoE genotype. (A) Astrocyte-enriched glial cultures from WT mice were treated with 2μM simvastatin for 96 hours with or without 10μM farnesyl pyrophosphate (FPP). Conditioned media (CM) was collected and extracellular ApoE levels were assessed by ELISA. N=5 biological replicates/condition, one-way ANOVA. **(B)** WT astrocyte-enriched glia were treated with 2μM simvastatin or simvastatin with the isoprenoids GGPP or GGOH at the indicated concentrations. Levels of ApoE in the CM were analyzed. N=2-3 biological replicates/condition, one-way ANOVA. **(C)** Astrocyte-enriched cultures from ApoE TR mice were treated with either 2μM simvastatin or simvastatin supplemented with 40μM GGOH or 20μM GGPP for 96 hours. CM and cell lysates were collected and ApoE levels were assessed by ELISA. CM ApoE levels were normalized to total cell lysate protein concentration. N=4-8 biological replicates/condition, one-way ANOVA. All data are normalized to controls and graphed as mean + s.e.m. CM, conditioned media; FPP, farnesyl pyrophosphate; GGOH, geranylgeraniol; GGPP, geranylgeranyl pyrophosphate; Simva, simvastatin.

2.4.5. Statins and prenylation inhibitors impair ApoE secretion, not reuptake

We have shown an important role for geranylgeranylation in modulating extracellular ApoE levels from astrocyte-enriched cultures from ApoE TR mice that is independent of ApoE genotype. Thus far, our experiments have been conducted with treatment lengths of 3-5 days in total. However, ApoE lipoparticles can bind to their receptors (particularly LRP1) on the cell surface, resulting in their being endocytosed[328]. After being endocytosed, ApoE particles may be degraded through the endolysosomal pathway or shuttled to recycling endosomes to be re-secreted[329]. It is unclear whether the observed impact on extracellular ApoE levels in our experiments using statins and prenylation inhibitors is the result of a specific modulation of ApoE secretion, reuptake, or both. Therefore, we sought to address these possibilities.

We pre-treated astrocyte-enriched glial cultures from WT cells with 10μg/mL cycloheximide (CHX) for 12 hours in order to inhibit protein synthesis and allow us to

better control when ApoE production could begin for the cells. After 12 hours, the CHX was washed off of the cells and replaced with DMSO, 2μM simvastatin, or 10μM FGTI 2734 each with either 200nM GST or 200nM of the LRP1 antagonist RAP-GST. We collected the conditioned media and cell lysates after 6 hours of treatment and assessed extracellular ApoE levels by ELISA. We found that RAP-GST treatment significantly increased extracellular ApoE levels in control cells, suggesting that RAP-GST was able to bind and antagonize LRP1 successfully, thereby blocking ApoE reuptake (Figure 2.5A). Both simvastatin and FGTI 2734 reduced ApoE levels in the CM and the addition of RAP-GST to either drug significantly elevated ApoE levels to the same extent as in the controls (Figure 2.5A). This suggests that inhibition of prenylation predominantly impacts ApoE secretion, and not reuptake. In support of this, extracellular ApoE content was significantly reduced in both simvastatin with RAP-GST and FGTI 2734 with RAP-GST conditions compared to controls with RAP-GST. This is likely due to there being less ApoE being released from the cells to begin with as a result of simvastatin and FGTI 2734 treatment. Taken together, our findings show that geranylgeranylation mediates proper secretion of ApoE independent of ApoE isoform, and that statins and prenylation inhibitors modulate secretion rather than reuptake of ApoE (Figure 2.5B).

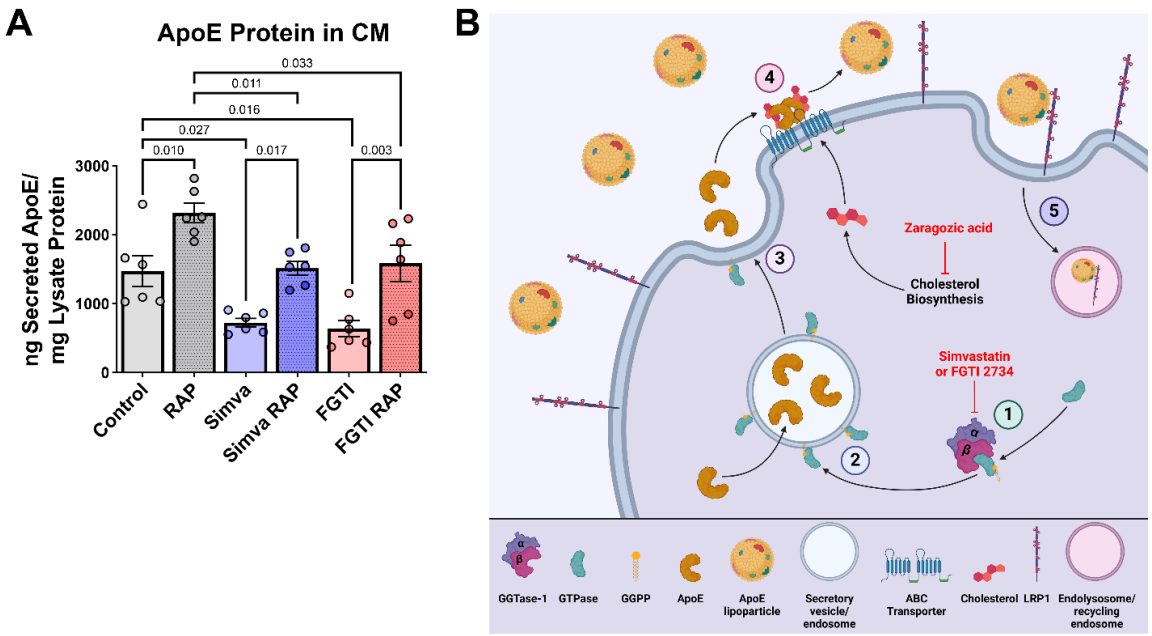


Figure 2.5. Simvastatin and FGTI 2734 impair ApoE secretion, not reuptake. (A) Astrocyte-enriched glial cultures from WT mice were pre-treated with 10µg/mL cycloheximide for 12 hours to inhibit protein synthesis. Cycloheximide was washed off and the cells were subsequently treated with DMSO, 2µM simvastatin, or 10µM FGTI 2734 each in the presence of either 200nM GST control or 200nM of the LRP1 antagonist RAP-GST. After 6 hours of treatment, both conditioned media (CM) and cell lysate was collected and extracellular ApoE was quantified by ELISA. Extracellular ApoE levels in the CM were normalized to total cell lysate protein. N=6 biological replicates/condition, two-way ANOVA. All data are presented as mean + s.e.m. B) Model depicting the impact of geranylgeranylation on ApoE secretion. Small GTPases get geranylgeranylated by GGTase-1 (1) allowing them to embed into membranes to conduct their function (2). This may be inhibited by statins or prenylation inhibitors. Some of these geranylgeranylated GTPases assist in trafficking secretory vesicles and endosomes that may contain ApoE to the cell surface for exocytosis (3). Once ApoE has been released, it remains attached to the extracellular matrix, where ABC transporters shuttle cholesterol and lipids onto ApoE to generate ApoE lipoparticles (4). These lipoparticles are released and may be endocytosed after binding to ApoE receptors such as LRP1. After endocytosis, ApoE lipoparticles may either be degraded in the endolysosome or shuttled to recycling endosomes to be re-secreted (5). Model was made using Biorender.com. CM, conditioned media; FGTI, FGTI 2734; GGPP, geranylgeranyl pyrophosphate; GGTase-1, geranylgeranyltransferase type 1; RAP, RAP-GST; Simva, simvastatin.

2.5. DISCUSSION

Here, we used specific inhibitors targeting multiple steps along the mevalonate pathway to determine how prenylation regulates ApoE secretion from ApoE TR astrocyte-enriched glia. We show that prenylation does modulate ApoE secretion from glia with human ApoE2, E3, and E4. Interestingly, we find that blocking FTase and GGTase-1 together has a similar effect between genotypes, and that giving back isoprenoid substrates for geranylgeranylation in the presence of simvastatin rescues extracellular ApoE levels for all ApoE isoforms. We also show that inhibiting FTase alone does not modulate ApoE secretion from cells with any of the ApoE genotypes, suggesting that the mechanism underlying the reported lonafarnib-mediated reductions in AD pathology in animal models[298] is independent of ApoE release. Interestingly, we find that the mechanism of ApoE secretion impairment induced by statins and prenylation inhibitors is due to their specific effect on secretion and not reuptake of ApoE (Figure 2.5A-B). Importantly, we show validation that the drugs used in the current study worked and inhibited their intended targets, providing stronger evidence of the observed effects compared to an earlier study[291]. Our results suggest a general role for prenylation in regulating secretion of ApoE, and a role for geranylgeranylation that is independent of ApoE isoform. These findings yield novel insights into the processes governing ApoE secretion that may be important considerations when generating therapies for modulating ApoE.

Peripheral ApoE is secreted through the canonical secretory pathway, beginning with its translocation into the ER, followed by its transport to the Golgi apparatus and trans-Golgi network before being loaded into vesicles and trafficked to the plasma membrane for secretion[330,331]. This process has yet to be fully observed and

characterized for brain-derived ApoE. In addition, depending on the cellular environment, ApoE may associate with other organelles, such as lipid droplets and mitochondria[147,332,333]. In macrophages, a number of modulators of ApoE secretion have been identified, including statins, intracellular cholesterol loading, protein kinase A, intracellular calcium levels, and possibly LDLR expression[331,334–337]. The endocytosis- and exocytosis-related dynamin proteins also appear to be involved in ApoE secretion from macrophages[338].

In the brain, regulation of ApoE secretion from microglia and astrocytes is altered by inflammation, ABCA1 expression and activity, lipolysis-stimulated lipoprotein receptor activity, and 25-hydroxycholesterol[120,146,148,339,340]. It is unknown if prenylation impacts or is impacted by any of these processes. Many of the proteins responsible for intracellular trafficking of proteins, endosomes, and secretory vesicles that may themselves carry ApoE are prenylated, including the geranylgeranylated proteins RhoA, Rac1, and Cdc42[341–347]. Therefore, we suspect that one potential mechanism for the role of geranylgeranylation in regulating ApoE secretion is by regulating GGTase-1-prenylated proteins. Indeed, a recent proteomic profiling of prenylated proteins in astrocytes confirms the expression and active prenylation of many of these GGTase-1 substrates in astrocytes[348].

Using inhibitors targeting various steps in the mevalonate pathway, we show that proper secretion of ApoE is dependent on prenylation. Our experiments using simvastatin and the dual prenylation inhibitor FGTI 2734 in combination with the LRP1 antagonist RAP were slightly surprising to us. We expected that prenylation inhibition would be important for both secretion and reuptake of ApoE given the importance of geranylgeranylation on the proper functioning of a variety of small GTPases involved in

the endocytic pathway. However, our data clearly suggest secretion is the primary process affected by these drugs. It is possible that geranylgeranylated GTPases involved in secretion are more directly involved in ApoE dynamics compared to those driving reuptake and therefore, our drug treatments predominantly impact secretion of ApoE. While this may be the case, additional experiments are needed to more specifically and definitively identify the mechanisms by which geranylgeranylation modulates ApoE secretion and dynamics.

2.5.1. Limitations

In the experiments presented here, we use astrocyte-enriched mixed glial cultures taken from humanized targeted-replacement mice harboring one of the human ApoE2, ApoE3, or ApoE4 genes in place of the mouse ApoE gene. While this can yield some insight as to how ApoE is regulated and whether it is regulated differently between genotypes, there are multiple other aspects modulating ApoE that cannot be accounted for in these mice. Specifically, the expression of the human ApoE genes is under the mouse ApoE promoter rather than the human promoter, which may yield different transcript levels compared to human cells. Additionally, how having human ApoE protein and mouse ABC transporters, LRP1, and LDLR impacts the kinetics of lipoparticle generation, receptor binding, and reuptake are not well-defined. While these factors may influence our results to some extent, in our model system, we are able to observe a significant role for geranylgeranylation in regulating ApoE secretion, suggesting that this is still a critical factor influencing ApoE biology.

GGTase-1 is thought to prenylate approximately 60 different proteins[349]. The current study did not set out to identify the specific GGTase-1 substrate or substrates that regulate proper ApoE lipoparticle secretion from astrocytes. Due to the promiscuity

of GGTase-1 and the ability of some prenylated proteins (namely, some forms of Ras) to be alternatively prenylated by GGTase-1 in the case of FTase inhibition, definitively elucidating those proteins involved with ApoE secretion may be challenging. This is especially true given the likelihood that multiple geranylgeranylated and prenylated proteins are imperative for this process. Nonetheless, clarifying these mediators of ApoE secretion may yield novel drug targets for modulating ApoE, particularly when considering the potential for targeting specific ApoE genotypes.

2.5.2. Conclusions

The data presented here show that geranylgeranylation is partly responsible for regulating secretion of ApoE in astrocyte-enriched cultures from ApoE TR mice, and that this is equally the case for all ApoE isoforms. Our data provide insight into the complexity of ApoE regulation and the potential for targeting prenylation for modulation of ApoE in AD. Future studies will need to be conducted to identify prenylated proteins involved in mediating proper ApoE secretion from glia and for further determining the molecular mechanisms by which statins and prenylation inhibitors affect ApoE biology. In addition, future experiments assessing whether prenylation is altered during AD in mice and humans who express the different human ApoE isoforms may lead to important understanding of the relationship between ApoE, prenylation, and AD pathogenesis and disease progression.

Chapter 3. Future Directions

3.1. Summary

The work in this dissertation has focused on the role of protein prenylation and the mevalonate pathway on ApoE secretion from glial cells harboring the three human ApoE isoforms. I found that GGTase-I-mediated geranylgeranylation mediates proper secretion of ApoE lipoparticles from glia, and that it does so independent of ApoE isoform. In this chapter, I will add to these findings by discussing alternative explanations for our findings presented thus far, as well as future directions and preliminary data that may expand our understanding of how the mevalonate pathway and other pathways regulate ApoE biology. Specifically, I will discuss recycling of ApoE lipoparticles and present preliminary data and propose future experiments that focus on the role cholesterol synthesis plays in modulating ApoE lipoparticle size and composition. I will subsequently describe how Rab-specific geranylgeranylation mediated by GGTase-II may be involved in allowing for normal ApoE lipoparticle secretion and how inflammation may regulate ApoE secretion independently from protein prenylation.

3.2. ApoE secretion and recycling

In Chapter 2, I showed that treating primary mixed glial cultures with statins and the dual FTase and GGTase-I inhibitor FGTI 2734 reduced conditioned media ApoE concentration no matter which ApoE isoform was expressed. Further, statin rescue experiments revealed geranylgeranylation is an important factor regulating ApoE lipoparticle secretion from glia independent of ApoE isoform. The finding that inhibiting prenylation resulted in a similar inhibition of ApoE secretion in all three genotypes (compared to baseline for each genotype) suggests that this mechanism is equally important for all genotypes. It also implies that the effect that prenylation has on

regulating ApoE secretion does not explain the differences in secretion between genotypes that I and others have shown in both *in vitro* and *in vivo* studies of mice and humans (Supplementary Figure 2.4)[66,120,129,130].

While it may be the case that ApoE secretion is regulated by prenylation, one of the results I presented in Chapter 2 was a bit surprising. Pre-treating cells with the protein synthesis inhibitor cycloheximide (CHX) and subsequently treating them with DMSO as a control, simvastatin, or FGTI 2734 for 6 hours revealed an expected reduction in extracellular ApoE content (Figure 2.5). However, when we added RAP to these treatments, it led to a relatively similar increase in extracellular ApoE in all conditions (Figure 2.5). RAP (receptor-associated protein) is an ER-resident chaperone protein that binds to the LDLR family of receptors in the ER and traffics them to the Golgi, where the lower pH leads to its dissociation from the receptors[350–355]. RAP is an antagonist of ligand binding to the LDLR family of receptors and is essential for receptor trafficking to the plasma membrane[352]. The goal of this experiment was to determine whether our drug treatments (simvastatin and FGTI 2734) were impacting ApoE secretion, reuptake and recycling, or both. The increase in conditioned media ApoE levels in DMSO-treated cells given RAP was not surprising as blocking reuptake of ApoE with RAP should lead to higher ApoE content. For simvastatin and FGTI 2734, however, I expected that our treatments were impacting both secretion and uptake and recycling.

As discussed in Chapter 1.2.2., much of the ApoE that is secreted by cells is then endocytosed and recycled in order to be re-secreted. Many of the proteins that mediate trafficking of proteins, endosomes, vesicles, and secretory granules are small GTPases in the Ras superfamily that require prenylation to conduct their function. Therefore, it is reasonable to assume that inhibiting prenylation of many of these small GTPases would

negatively impact exocytosis, endocytosis, and recycling of ApoE lipoparticles. But surprisingly, our data showed exocytosis is the most impacted process from our drug treatments. One potential explanation for this might be that prenylated proteins involved in ApoE dynamics are more prominent in the secretion of the apolipoprotein compared to those that mediate uptake and recycling. Preliminary findings suggest a potential role for ER-Golgi trafficking. These and data that may implicate a still-important role for recycling will be discussed, as well as additional experiments that may be done in the future to further understand the role of prenylation in ApoE dynamics.

3.2.1. Preliminary data

The secretory pathway begins with newly-synthesized proteins being imported into the ER, where they are folded and processed accordingly. Proteins destined for secretion can then be transported to the ER-Golgi intermediate complex (ERGIC) through Coat Protein Complex II (COPII)-mediated budding of ER vesicles that are shuttled to the ERGIC. The cargo then goes through the Golgi apparatus and trans-Golgi network (TGN), and is subsequently packaged into secretory vesicles that are transported to the plasma membrane for exocytosis. The data presented in Chapter 2 showed that geranylgeranylation is important for proper secretion of ApoE lipoparticles from glia. However, we did not identify an exact mechanism describing how geranylgeranylation mediates ApoE secretion or what steps in the secretory pathway are negatively impacted by simvastatin and FGTI 2734 treatment.

In order to begin to identify these mechanisms in our cell culture system, I treated astrocyte-enriched mixed glial cultures from the cortex of wild-type (WT) mice with DMSO as a control, 2μM simvastatin, and 10μM FGTI 2734 for 96 hours. Following drug treatment, I collected the conditioned media (CM) and cell lysate and a post-doctoral

fellow in our lab (Dr. Seyyedmohsen Hosseinibarkooie) conducted tandem-mass-tag mass spectrometry (TMT-MS) on the cell lysate samples. Analysis showed that simvastatin treatment significantly reduced the amount of the prenylated proteins Rap1a and Lamin B2 (Figure 3.2.1A), as well as proteins that require prenylated proteins to function, such as Ipo5, which facilitates nuclear protein import through a Ran-dependent mechanism (Figure 3.2.1B)[356]. In addition, there were a number of significantly different proteins that, in some way, work alongside small GTPases to conduct their function. These include proteins that are involved in or proposed to be involved in a variety of processes, including regulation of or by small GTPases, receptor trafficking/scaffolding, ER function, and breakdown of trafficking components (Figure 3.2.1B). Importantly, both simvastatin and FGTI 2734 altered the amount of several proteins involved in various steps along the secretory pathway, particularly ER-to-Golgi transport (Figure 3.2.1C). Of note, the proteins Tmed2 and Tmed10, which act as receptors that assist in loading COPII vesicles with cargo for transport to the Golgi[357], were significantly altered. Interestingly, these proteins are also important for cholesterol and ceramide transfer at ER-Golgi contact sites and modulate plasma membrane lipid nanodomains as a result[358]. Simvastatin treatment also increased the protein levels of Surf4, which is imperative for secretion of the LDLR-regulating glycoprotein PCSK9, as well as secretion of VLDL[359]. The observed increase in major components of the secretory pathway in response to simvastatin and FGTI 2734 treatment imply a compensatory upregulation of protein trafficking and possibly secretion. This could suggest that inhibiting protein prenylation impairs the homeostatic functioning of the secretory pathway, thereby explaining our observed effects on ApoE secretion. Nonetheless, additional experiments are required in order to validate and better understand the molecular mechanisms

underlying the role of geranylgeranylation in shuttling ApoE through the secretory pathway for secretion.

A

Decreased Prenylated Proteins

Protein	Abundance Ratio: Simva/DMSO	p-Value
Rap1a	0.124	2.603×10^{-9}
Lmnb2	0.165	1.287×10^{-6}

B

Increased GTPase-Related Proteins

Protein	Abundance Ratio: Simva/DMSO	p-Value	Protein	Abundance Ratio: FGTI/DMSO	p-Value
Nherf2	6.863	1.717×10^{-15}	Rcn3	2.04	2.221×10^{-2}
Rasal1	1.882	1.790×10^{-3}	Cd151	2.051	3.261×10^{-2}
Gna12	1.478	3.344×10^{-2}	Hspa8	2.158	4.195×10^{-2}
			Pea15	1.912	5.273×10^{-2}

Decreased GTPase-Related Proteins

Protein	Abundance Ratio: Simva/DMSO	p-Value	Protein	Abundance Ratio: FGTI/DMSO	p-Value
Ipo5	0.010	1.717×10^{-15}	Dlg1	0.502	2.496×10^{-3}
Synpo2	0.084	9.356×10^{-14}			
Cse1l	0.185	1.050×10^{-5}			
Nmt1	0.259	2.387×10^{-3}			
Ssr1	0.274	4.776×10^{-3}			
Ppp1r12a	0.274	4.776×10^{-3}			
Farp1	0.321	3.130×10^{-2}			

C

Secretory Proteins

Protein	Abundance Ratio: Simva/DMSO	p-Value	Protein	Abundance Ratio: FGTI/DMSO	p-Value
Golph3	4.548	4.212×10^{-11}	Tmed10	2.233	2.735×10^{-2}
Emc2	2.894	1.351×10^{-6}	Arf3	2.189	3.385×10^{-2}
Stxbp2	2.033	6.218×10^{-4}			
Tmed2	1.732	5.232×10^{-3}			
Surf4	1.661	9.101×10^{-3}			
Arl3	1.614	1.293×10^{-2}			
Anxa7	1.432	4.619×10^{-2}			
Uso1	0.205	6.470×10^{-5}			
Scrn1	0.298	1.391×10^{-2}			
Dnajb11	0.320	3.046×10^{-2}			

D

Endocytic Proteins

Protein	Abundance Ratio: Simva/DMSO	p-Value	Protein	Abundance Ratio: FGTI/DMSO	p-Value
Chmp4b	2.103	3.808×10^{-4}	Pfn1	2.619	2.368×10^{-3}
Pdcd6ip	1.768	3.978×10^{-3}			
Hspa12a	0.178	5.623×10^{-6}			
Twf1	0.238	7.452×10^{-4}			
Snx3	0.336	4.857×10^{-2}			

E

Increased Tubulin Proteins

Protein	Abundance Ratio: FGTI/DMSO	p-Value
Tubb5	2.793	7.646×10^{-4}
Tubb4b	2.516	4.494×10^{-3}
Tubb2a	2.266	2.194×10^{-2}
Tubb1a	2.176	3.665×10^{-2}

Figure 3.2.1. Simvastatin and FGTI 2734 affect both secretory and endocytic pathways after longer treatment. Astrocyte-enriched glial cultures from WT mice were treated with either DMSO, 2μM simvastatin, or 10μM FGTI 2734 for 96 hours. Cell lysate was collected and processed for TMT-MS analysis. Abundance ratios and p-values of significantly changed prenylated proteins **(A)**, GTPase-related proteins **(B)**, secretory proteins **(C)**, endocytic proteins **(D)**, and tubulin proteins **(E)** are presented. N=3 independent experiments/condition, FDR-adjusted p-values are shown. FGTI, FGTI 2734; Simva, simvastatin. TMT-MS data was collected and analyzed by Dr. Seyyedmohsen Hosseinibarkooie.

Prenylation is imperative for the proper function of proteins that modulate cell trafficking along the secretory pathway. However, prenylated small GTPases are also imperative for the various endocytic pathways. Indeed, in the same TMT-MS dataset we collected where we observed altered levels of proteins involved in ER-to-Golgi transport after simvastatin and FGTI 2734 treatment, we also identified differences in proteins associated with endocytic trafficking (Figure 3.2.1D). We found changes in proteins regulating endosomal sorting into recycling endosomes and late endosomes presumably destined for lysosomal degradation. Most notably, simvastatin increased levels of Chmp4b and its adapter protein Pdcd6ip, which are imperative for the endosomal sorting complex ESCRT-III[360]. At the same time, simvastatin decreased Hspa12a and Snx3, which modulate endosomal sorting and recycling[361,362], as well as Twf1, which is involved in clathrin-mediated endocytosis[363]. We also identified multiple forms of actin whose protein levels were changed in response to drug treatment (Figure 3.2.1E), which is noted due to the important role of actin filaments in mediating trafficking and endocytosis. Therefore, our mass spectrometry data suggests a potential impact of prenylation inhibition in receptor-mediated endocytosis and recycling.

Altogether, my preliminary findings in astrocyte-enriched mixed glial cultures show that both simvastatin and FGTI 2734 altered levels of proteins essential for the

secretory pathway and endocytosis. These data suggest a possible importance for geranylgeranylation on ApoE secretion, as well as uptake and recycling. However, in light of my results in Figure 2.5 showing modulation of secretion and not uptake by simvastatin and FGTI 2734, additional experiments are required to better determine the role of geranylgeranylation in ApoE dynamics.

3.2.2. Future directions

The first critical step that I would take for future directions would be to validate our mass spectrometry data with WBs measuring levels of secretory and endocytic pathway proteins that were changed by simvastatin and FGTI 2734. My preliminary data showing simvastatin and FGTI 2734 likely impact endocytosis and possibly recycling is potentially in contrast to the data that I observed in Figure 2.5 in Chapter 2, which showed that secretion of ApoE was the predominant factor being impacted. One explanation for this discrepancy is that there is a temporal effect, as the TMT-MS experiment was of cells treated for 96 hours while the RAP experiment in Figure 2.5 was only 6 hours of treatment. Therefore, it may be the case that the secretory pathway is impacted by simvastatin and FGTI 2734 first and the changes in the endocytic and secretory pathways arise after a longer period of inhibition. It may also be possible that the endocytic pathways that these drugs impair are not involved in ApoE uptake and recycling, thereby explaining why only secretion of ApoE appeared to be affected. Nonetheless, conducting additional experiments to better characterize the role of geranylgeranylation on ApoE dynamics may yield significant findings into the regulation of ApoE.

In order to determine the fate of ApoE in response to simvastatin and FGTI 2734, I would treat primary astrocyte-enriched mixed glial cultures from wild-type (WT) mice

with these drugs and conduct immunocytochemistry (ICC) staining for ApoE and the astrocyte marker GFAP. In addition, staining for various cellular compartments would be highly beneficial in order to determine where ApoE may be pooled within the cell, if at all, as a result of drug treatment. Specifically, I would stain for calreticulin in the ER, GM130 for the Golgi, Rab5 for early endosomes, Rab7 for late endosomes, and Lamp1/2 for lysosomes. There are also a number of organelle stains that work well for staining these compartments in both live and fixed cells that could be used. If I were to conduct this experiment, I may expect to find more ApoE targeted to the ER and lysosome in simvastatin- and FGTI 2734-treated cells based on our mass spectrometry data. However, as there are also a number of proteins involved in trafficking through the Golgi that were impacted by these drug treatments in our results, there may be a buildup of ApoE in the Golgi that is unable to be trafficked to the plasma membrane for secretion as well. This is one method for determining the effect of prenylation inhibition on ApoE dynamics, but there are other methods that I have begun to work on that may yield more insight into this.

Our lab recently received three plasmids containing enhanced green fluorescent protein (EGFP) attached to the N-terminus of ApoE2, ApoE3, and ApoE4 from Dr. Brad Hyman's lab at Massachusetts General Hospital. Their group developed these plasmids in order to measure ApoE-Aβ interactions and validated its ability to accurately assess ApoE[267]. I am currently in the process of growing up more of these plasmids and will be incorporating them into lentiviral vectors. The reason for putting them into a lentivirus is because of our experience and that of others finding low transfection efficiency of plasmids into brain cells using classical delivery methods, such as lipofectamine. Likewise, both astrocytes and neurons in culture are amenable to lentiviral transduction.

Therefore, incorporating the ApoE-EGFP constructs into lentiviruses will allow for the most potential use cases for future experiments. Once we have made and validated the ApoE-EGFP lentiviral vectors, we will be able to utilize them to better address the question of how statins and prenylation inhibitors impact ApoE secretion, uptake, and recycling.

To do so, I would culture astrocyte-enriched mixed glial cultures from ApoE knock-out (KO) mice and transduce them with our ApoE-EGFP lentiviruses in order to visualize ApoE inside and outside the cell. I would first validate that our cells were transduced as expected by imaging the cells either alive or fixed, and by collecting cell lysate to measure EGFP and ApoE protein on a WB (Figure 3.2.2). After treating the cells with DMSO as a control, 2μM simvastatin, and 10μM FGTI 2734 for 24 hours or so, I would use live-cell ER, Golgi, endosome, and lysosome stains to colocalize ApoE with these compartments. From here, I would do either live-cell confocal microscopy or fix the cells and stain them for GFAP to denote astrocytes before imaging. This may allow us to better characterize ApoE dynamics in live cells and with better accuracy than immunostaining.

To assess ApoE lipoparticle uptake by glia treated with our inhibitors, I would first transduce primary astrocyte-enriched mixed glial cultures from ApoE KO mice with each of our three ApoE isoform lentiviruses. The CM from these transduced cells will contain ApoE-EGFP lipoparticles that I would then collect for additional experiments. To test how statins and prenylation inhibitors impact ApoE uptake, I would pre-treat non-transduced ApoE KO glia with DMSO, 2μM simvastatin, 1μM zaragozic acid, or 10μM FGTI 2734 for 3-6 hours and then add the concentrated ApoE-GFP CM from the transduced cells. The ApoE-GFP-containing CM would be incubated on the cells for another 3-6 hours before

collecting the resulting CM, and either collecting the cell lysate to conduct WBs for ApoE and GFP protein or fix the cells to image intracellular ApoE-GFP signal (Figure 3.2.2). Comparing levels of intracellular ApoE-GFP will allow us to determine how much our drug treatments impact ApoE lipoparticle uptake, and also whether there is a difference in the amount of uptake based on ApoE isoform. I would expect to find impaired ApoE lipoparticle uptake in cells treated with simvastatin and FGTI 2734 based on our preliminary TMT-MS results.

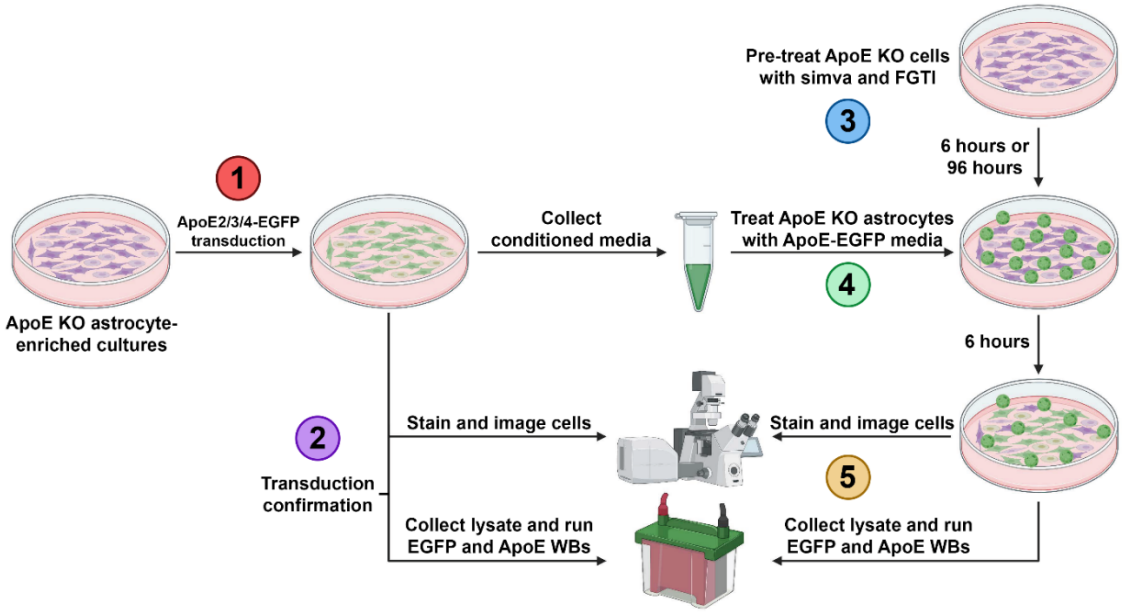


Figure 3.2.2. Treatment plan for human ApoE-EGFP lentivirus experiments. (1) Astrocyte-enriched glial cultures from ApoE knockout (KO) mice would transduced with human ApoE2/3/4-EGFP lentiviral vectors. **(2)** To confirm transduction was successful, cells will be fixed and imaged by confocal microscopy and cell lysate will be collected to be run on a western blot (WB) for EGFP and ApoE protein. **(3)** Separate primary astrocyte-enriched cultures from ApoE KO mice that have been pre-treated with statins and prenylation inhibitors will be treated with ApoE-EGFP conditioned media **(4)**. **(5)** ApoE-EGFP uptake and recycling will be measured by confocal microscopy, and by WB for EGFP and ApoE protein in the cell lysate and conditioned media.

We can also utilize the ApoE-EGFP lentiviruses to measure ApoE lipoparticle recycling in our system. To do so, I would conduct a similar experiment as the one described above for measuring ApoE lipoparticle uptake. However, I would not pre-treat the non-transduced ApoE KO cells with our drugs and instead, give them the drugs at the same time as the concentrated ApoE-EGFP CM in order to not pre-emptively impair ApoE uptake due to drug treatment. After either 6 or 96 hours of treatment, I would wash off the ApoE-EGFP CM containing our drugs of interest and replace it with regular serum-free media with the drugs. I would leave these on the cells for 3-6 more hours and then collect the conditioned media from these cells and run WBs and ELISAs for ApoE and EGFP in order to determine how much ApoE was re-secreted from the cells between our drug treatments. Because there were proteins involved in receptor endocytosis and recycling that were changed in response to simvastatin and FGTI 2734 in our TMT-MS results, I would hypothesize to see a reduced amount of re-secreted ApoE in the CM of cells given both of these drugs compared to controls.

Another experiment I believe would greatly increase our understanding of the mechanisms mediating proper ApoE secretion, reuptake, and recycling would be to conduct immunoprecipitation (IP) experiments in transduced ApoE KI cells treated with our inhibitors. Following ApoE IP, I would run TMT-MS for ApoE binding partners in controls and determine which of these are altered in response to statin or prenylation inhibitor treatment. The presence of an EGFP added to the ApoE makes IPs much easier and more reliable to conduct. Indeed, I performed preliminary experiments of ApoE IP after DMSO, simvastatin, and zaragozic acid treatment in WT astrocyte-enriched mixed glial cultures followed by TMT-MS conducted by Dr. Hosseinibarkooie. While we were able to see a decent immunoprecipitation of ApoE in our control cells

compared to non-IP control cells, it was not as good and did not yield as clean of a dataset as we would have liked. Therefore, I believe that being able to IP EGFP instead of ApoE itself would lead to greater IP efficiency and better, more reliable TMT-MS results.

Lastly, it would be highly beneficial to characterize the prenylome of astrocytes derived from ApoE2, E3, and E4 TR mice. In Chapter 2, I showed that inhibition of prenylation impacted ApoE release from astrocytes equally in all human ApoE genotypes. However, it is possible that there are differences in the extent of prenylation, as well as which proteins are preferentially prenylated in these different ApoE cells. To my knowledge, this has not been assessed yet, although some have begun to characterize the astrocyte prenylome[348]. To test this, there have been a number of different probes developed for quantifying levels of prenylated proteins by mass spectrometry[296,299,348,364]. Utilizing one of these prenylation probes to determine potential differences in the astrocyte prenylome between ApoE genotypes may yield novel insights into whether there is variation in the prenylation of Ras superfamily proteins that are involved in vesicular and endosomal trafficking at baseline. Further, conducting the same experiment with astrocytes from ApoE TR mice crossed with an AD mouse model or measuring the total prenylome of the brains of these mice may provide additional important information regarding how the endolysosomal system is impacted in the context of AD.

The experiments proposed here would significantly improve our understanding of ApoE dynamics and the role that prenylation plays in regulating ApoE, thereby potentially shedding light on specific mechanisms and proteins in the secretory and endolysosomal systems that may be targeted for therapeutic ApoE modulation.

3.3. Geranylgeranylation of Rab GTPases may modulate ApoE secretion

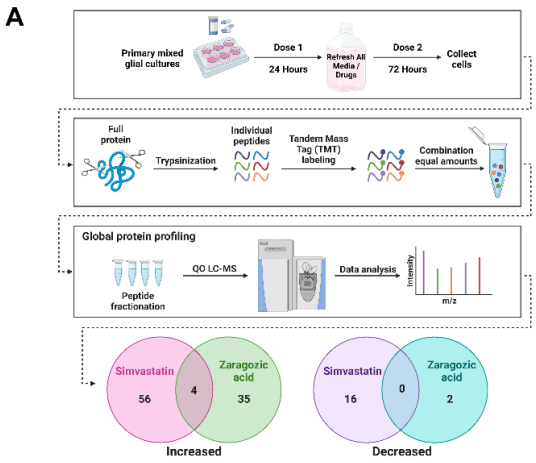
Thus far, most of the data presented and discussed throughout Chapters 2 and 3 have focused on prenylation mediated by either FTase or GGTase-1. Indeed, I interpreted our data showing a rescue of the simvastatin-induced impairment to ApoE secretion with the geranylgeranylation substrate GGPP as likely due to its effects on GGTase-1. While I believe this is an argument supported by the fact that FGTI 2734 also impairs ApoE secretion and is a specific inhibitor of only FTase and GGTase-1, there is still the possibility that geranylgeranylation by other GGTases may be involved in regulating ApoE dynamics. Rab-specific geranylgeranylation by GGTase-II is the most likely candidate for this, as Rab proteins are the largest subgroup of the Ras superfamily of proteins and are known to be imperative for intracellular trafficking of cargo[365]. Additionally, I have generated preliminary data implicating Rab proteins as being modulated by statin treatment. These and future experiments to further investigate the role of Rab proteins on ApoE dynamics will be discussed in this section.

3.3.1. Preliminary data

There are many key components allowing for intracellular trafficking of proteins, vesicles, and organelles. This is particularly important for cells such as neurons, whose long axons and great energetic demands require a great amount of cargo shuttling between soma and synapses. Microtubules and the cytoskeleton act as the foundation for this process, while a large array of proteins are the drivers of cargo shuttling. Of these, proteins in the Rab family of small GTPases are essential mediators of vesicle trafficking. There have been over 70 different Rab proteins identified in humans, with many critically involved in the formation, transport, and target membrane fusion of

vesicles[365–368]. Changes in Rab geranylgeranylation can greatly influence cellular dynamics and health due to their involvement in trafficking of a wide variety of cargo, as well as their importance in cell signaling[365].

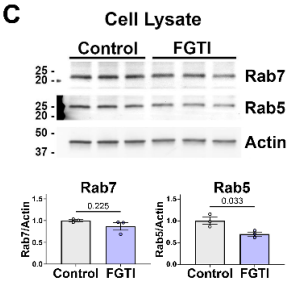
In order to get a better understanding of the mechanisms underlying our simvastatin effect on extracellular ApoE, I conducted experiments as outlined in section 3.2 where I treated primary WT astrocyte-enriched mixed glial cultures with DMSO as a control, 2μM simvastatin, or 1μM zaragozic acid for 96 hours. I subsequently collected the cell lysate and conditioned media from these cells and Dr. Hosseinibarkooie, a postdoc in our lab and expert in mass spectrometry, ran TMT-MS on the collected lysate samples. This experiment was actually the first of these mass spectrometry experiments we conducted and the results of this run were particularly striking. We identified 56 significantly increased and 16 significantly decreased proteins unique to simvastatin treatment, 35 increased and 2 decreased proteins specific for zaragozic acid treatment, and 4 proteins upregulated by both drugs (Figure 3.3.1A). Of particular interest were the 16 proteins significantly decreased by simvastatin alone for a couple of reasons. First, because they were impacted by only simvastatin and not zaragozic acid, it suggested that these proteins were altered due to simvastatin's effect on the prenylation arm of the mevalonate pathway, as inhibiting the cholesterol synthesis arm did not affect them. Second, this group stood out amongst the rest because 11 out of the 16 proteins significantly reduced by simvastatin were Rab proteins (Figure 3.3.1B).

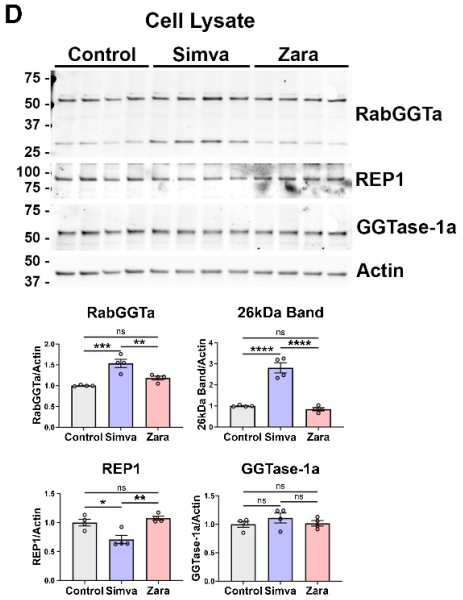


B

Decreased Proteins

Protein	Abundance Ratio: Simva/DMSO	p-Value
Rab7a	**0.653**	**4.679×10^{-7}**
Ctsd	0.670	1.813×10^{-6}
Rab5a	**0.683**	**1.638×10^{-3}**
Rab5c	**0.773**	**4.164×10^{-3}**
Rab10	**0.791**	**4.326×10^{-3}**
Rab4b	**0.673**	**5.030×10^{-3}**
Aldh3b1	0.773	1.197×10^{-2}
Cox5a	0.807	1.197×10^{-2}
Rab11b	**0.799**	**1.382×10^{-2}**
Rab1a	**0.801**	**1.440×10^{-2}**
Rab5b	**0.692**	**1.759×10^{-2}**
Rab2a	**0.809**	**2.221×10^{-2}**
Cox6b1	0.808	3.451×10^{-2}
Rab1b	**0.810**	**3.505×10^{-2}**
Mcfd2	0.734	4.077×10^{-2}
Rab6a	**0.826**	**4.466×10^{-2}**





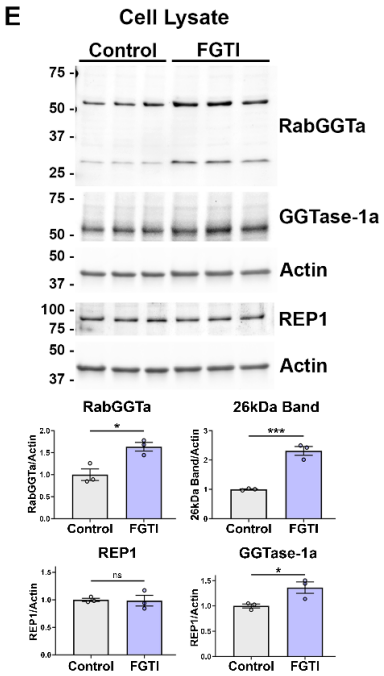


Figure 3.3.1. Simvastatin and FGTI 2734 impact Rab proteins and GGTase-II. (A) Astrocyte-enriched glial cultures from WT mice were treated with either DMSO, 2μM simvastatin, or 1μM zaragozic acid for 96 hours. Cell lysate was collected and processed for TMT-MS analysis. N=3 independent experiments/condition. **(B)** Table of identified proteins decreased only by simvastatin treatment from the experiment in (A). N=3 independent experiments/condition. Abundance ratios and FDR-adjusted p-values are shown. **(C)** Astrocyte-enriched mixed glial cultures from WT mice were treated with DMSO or 10μM FGTI 2734 for 96 hours. Lysate was collected and run on a WB for Rab7 and Rab5 levels. N=3 independent experiments, Student's t-test. **(D)** WT astrocyte-enriched cultures were treated with DMSO, 2μM simvastatin, or 1μM zaragozic acid for 96 hours. Cell lysate was collected and run on a WB to assess RabGGTa, REP1, and GGTase-1a protein levels. N=4 independent experiments/condition, one-way ANOVA. **(E)** Primary WT astrocyte-enriched cultures were treated with DMSO or 10μM FGTI 2734 for 96 hours and protein levels of RabGGTa, REP1, and GGTase-1a in the cell lysate were measured. N=3 independent experiments/condition, Student's t-test. FGTI, FGTI 2734; Simva, simvastatin; Zara, zaragozic acid. TMT-MS was performed and analyzed by Dr. Seyyedmohsen Hosseinibarkooie.

The Rabs identified in this dataset are essential mediators of cellular trafficking and transport in the secretory and endocytosis pathways, as well as recycling. Specifically, the proteins Rab1, Rab2, Rab6, Rab10, and Rab11 are all involved in shuttling cargo along the secretory pathway[365,369,370]. Rab7 is a key marker of late endosomes and is associated with the degradative pathway[371]. It also interacts with Rab5, a marker for early endosomes[372,373] that also works in tandem with Rab4 and Rab11, and these three combined are themselves involved in mediating recycling of endosomes[365,374–377]. The finding that all of these proteins were significantly decreased by simvastatin treatment in our cells suggests that Rab-dependent trafficking of vesicles and endosomes is strongly impaired by statins and that this may partly explain our observed effects on ApoE dynamics.

In addition, I treated cells with DMSO or 10μM FGTI 2734 for 96 hours and WBs were run on the cell lysate for Rab5 and Rab7 protein levels. I found a modest reduction

in Rab5 and not Rab7 protein in FGTI 2734-treated cells compared to controls (Figure 3.3.1C). I suspect the reason for the lack of statistically significant difference in Rab7 levels was due to the difference in methodology, as mass spectrometry is much more sensitive than WB. However, in future runs of TMT-MS that were described above in WT astrocyte-enriched glial cultures treated with DMSO, simvastatin, or FGTI 2734 for 96 hours, we found that in simvastatin-treated cells alone, only Rab5 and Rab1 were decreased (Figure 3.3.2). This may be due to increased variability in the samples between runs or another factor, but the fact that at least some of these proteins were reproducibly impacted suggests that this is a consistent effect that may be important to ApoE biology.

Decreased Rab Proteins

Protein	Abundance Ratio: Simva/DMSO	p-Value
Rab5a	0.221	2.495×10^{-4}
Rab1b	0.230	4.384×10^{-4}

Figure 3.3.2. Simvastatin decreases Rab protein expression. Astrocyte-enriched glial cultures from WT mice were treated with either DMSO, 2μM simvastatin, or 10μM FGTI 2734 for 96 hours. Cell lysate was collected and processed for TMT-MS analysis. Simvastatin treatment reduced protein levels of Rab5a and Rab1b. N=3 independent experiments/condition. Abundance ratios and FDR-adjusted p-values are shown. Simva, simvastatin. TMT-MS was performed and analyzed by Dr. Seyyedmohsen Hosseinibarkooie.

Rab proteins require geranylgeranylation by GGTase-II, also referred to as RabGGTase due to the enzyme's specificity for Rab proteins, in order to function properly. This process requires either REP1 or REP2, as they bind to non-prenylated

Rabs in the cytoplasm, mediate their binding and prenylation by GGTase-II, and ensure their subsequent delivery to their target membranes (see Chapter 1.3.3). Because simvastatin, but not zaragozic acid, should in theory be modulating prenylation substrate levels and appeared to impair Rab protein levels, I sought to determine whether parts of the Rab prenylation machinery were also altered in response to simvastatin. To do so, I treated WT astrocyte-enriched primary cultures with DMSO, simvastatin, and zaragozic acid for 96 hours. I collected the lysate from these cells and WBs were run for the GGTase-II subunit RabGGTa, REP1, and the α subunit of GGTase-1. This revealed a significant increase in the amount of RabGGTa and a significant decrease in REP1 in simvastatin-treated cells compared to controls, while zaragozic acid had no effect (Figure 3.3.1D). GGTase-1a was not altered in either drug treatment condition. Interestingly, I observed a consistent ~26kDa band when immunoblotting for RabGGTa that coincided with the increase in the band at the predicted molecular weight of RabGGTa at ~60kDa (Figure 3.3.1D). I am unsure what this band is exactly, but future experiments using a blocking peptide for the RabGGTa antibody may be beneficial in confirming that this is not some product of RabGGTa and that the ~60kDa band is in fact measuring RabGGTa as expected. Lastly, I wanted to determine whether FGTI 2734 might impact RabGGTa or REP1 levels as simvastatin did. After 96 hours of DMSO or 10μM FGTI 2734 treatment, I ran WBs on the cell lysate of these samples to determine whether these proteins were affected. Strangely, I still observed an increase in RabGGTa levels, but did not find any significant difference in REP1 protein (Figure 3.3.1E). Importantly, however, GGTase-1a levels were significantly increased. These results potentially suggest that inhibition of FTase and GGTase-1 with FGTI 2734 leads to a compensatory upregulation of GGTase-1 and GGTase-II in order to utilize the pool of GGPP being made by the cells and not used for geranylgeranylation by GGTase-1.

Altogether, these preliminary data suggest that Rab prenylation and function is impaired by statins and prenylation inhibitors. While this is not extremely surprising given the known importance of prenylation on Rab function, the potential for their influence on ApoE dynamics is interesting.

3.3.2. Future directions

There are a number of ways that one could determine the role of Rabs and their prenylation on ApoE dynamics. For one, there have been a number of GGTase-II inhibitors developed and these could be used to determine their effect on ApoE secretion, reuptake, and recycling. I have attempted to test this using a couple of different GGTase-II inhibitors. Unfortunately, I have not had much success in getting them to work in our system (as assessed by a Rab prenylation assay). As such, I propose that one method that could be used for this would be to use siRNAs targeting *Rep1*. I have conducted initial experiments using REP1 siRNAs to impair prenylation and shuttling of Rab proteins using Qiagen's FlexiTube siRNA system that comes with small aliquots of 4 siRNAs for your gene target of interest. I complexed each of the siRNAs and a scramble siRNA with either lipofectamine 3000 or Qiagen's HiPerfect Transfection Reagent and incubated them on WT astrocyte-enriched mixed glial cultures. Again, unfortunately, I was unable to see any difference in the amount of REP1 protein on a WB for any of the 4 siRNAs I received using either lipofectamine or HiPerfect Transfection Reagent. I do believe that this would be an ideal method for testing whether Rabs are important for ApoE secretion, uptake, and recycling, but it requires more optimization in order to work in our system. Another option for siRNA targeting for this question would be the GGTase-II subunit *Rabggta*, as it is unique to GGTase-II while the β subunit is not. After optimization of these siRNAs and confirmation that they worked as intended, I

would collect conditioned media from cells treated with no siRNA and scramble siRNA as controls, as well as the optimized siRNA targeting REP1 or RabGGTa and assess whether extracellular ApoE levels were impaired as a result of knockdown. Based on my preliminary data, I would hypothesize a potential impact on extracellular ApoE levels, but if this was the case, more experiments would need to be conducted to determine whether Rabs are imperative for ApoE secretion, uptake, recycling, or all of these aspects of ApoE dynamics.

Additionally, I would utilize the data from the experiment proposed at the end of section 3.2.2 in which I would transduce ApoE KO glia with the human ApoE-EGFP lentiviruses, treat them with DMSO, simvastatin, or FGTI 2734, and conduct EGFP immunoprecipitations followed by TMT-MS to determine ApoE binding partners and how they change in response to statin and prenylation inhibitor treatment. It may be the case that ApoE is able to bind to Rab proteins directly, and that they can assist in shuttling ApoE or ApoE lipoparticles through the secretory and endocytic pathways. If this is true, they may show up as binding partners that reduce their association with ApoE after simvastatin and FGTI 2734 treatment. If so, I would confirm these TMT-MS findings by conducting the same IP experiment, and running the precipitate on a WB for identified target binding partners. These experiments may provide additional insight into the mechanisms mediating ApoE dynamics and determine whether Rab prenylation is imperative for regulating proper ApoE shuttling and secretion. Furthermore, which Rab proteins may be identified as ApoE binding partners could guide future experiments for assessing the role Rabs have in proper ApoE secretion, uptake, and recycling.

3.4. Regulation of ApoE lipoparticle size and composition by cholesterol

ApoE plays an integral role in regulating cholesterol homeostasis throughout the body and in the brain. As discussed in Chapter 1, once it has been released onto the extracellular side of the cell surface, ApoE is lipidated by ABC transporters that transfer phospholipids and cholesterol onto ApoE[148–153]. This leads to the generation of ApoE lipoparticles that carry a variety of lipid species, but are predominantly comprised of phospholipids, cholesterol, and cholesterol esters[155]. Research suggests that lipoparticles of the three human ApoE isoforms have different lipid profiles and sizes[66], indicating a difference in the regulatory mechanisms of ApoE lipoparticle generation. The importance of this is clear to some extent, as lipid composition of ApoE lipoparticles appears to have a profound impact on cell biology and health in the brain. For example, when astrocytes are activated by proinflammatory stimuli, they secrete ApoE particles carrying long-chain saturated fatty acids that are toxic to neurons and oligodendrocytes, and this may contribute to neurodegenerative diseases[51]. While the data I have presented thus far has shown no role for cholesterol biosynthesis in regulating ApoE secretion, preliminary data that we have generated clearly suggest that cholesterol biosynthesis is imperative for proper ApoE lipoparticle production. In the following sections, I will discuss this data and experiments to conduct in the future to better understand the role of cholesterol biosynthesis in ApoE lipoparticle composition and production, as well as how this may be important for Alzheimer's Disease (AD).

3.4.1. Preliminary data

The initial data of the project that would become the published paper presented in Chapter 2 was collected by a former post-doctoral fellow in our lab, Dr. Josh Kulas. He

had found that, in WT astrocyte-enriched glial cultures, simvastatin, but not zaragozic acid reduces extracellular ApoE levels in a dose-dependent manner (Figure 2.1). Around the same time, he conducted experiments using the same cells treated with set concentrations of these drugs, but instead of looking at ApoE on a SDS-PAGE immunoblot, he decided to assess whether mevalonate pathway inhibition impacted another aspect of ApoE biology: lipoparticle size. To do so, he conducted native-PAGE of the conditioned media samples from cells treated with DMSO, 2μM simvastatin, or 1μM zaragozic acid for 96 hours. When he did so, he found the expected reduction in the high molecular weight (MW) ApoE band present in the control, but interestingly, he also found that zaragozic acid led to a significant reduction in the size of the ApoE lipoparticle (Figure 3.4.1A).

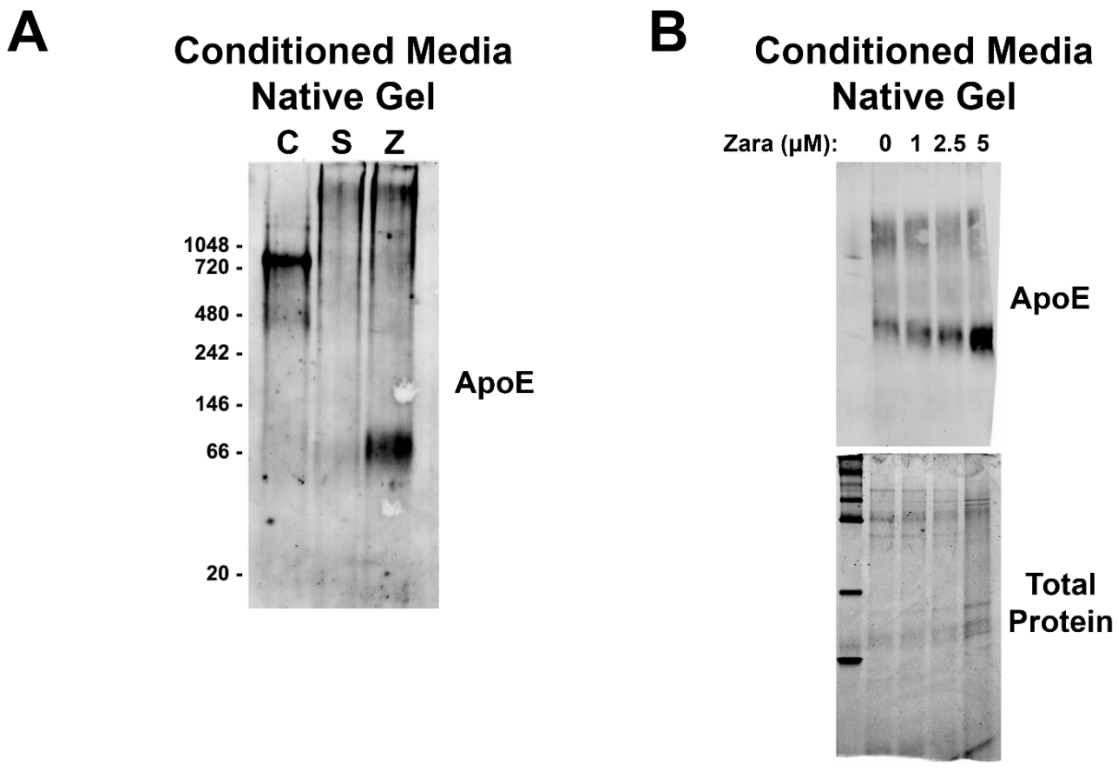


Figure 3.4.1. Inhibition of cholesterol synthesis leads to a dose-dependent reduction in ApoE lipoparticle size. (A) Astrocyte-enriched glial cultures from WT mice were treated with either DMSO, 2μM simvastatin, or 1μM zaragozic acid for 96 hours. Conditioned media (CM) was collected and concentrated using 10kDa MW cutoff centrifugation filters. Concentrated CM was run by native-PAGE and immunoblotted for ApoE. **(B)** WT cultures were treated with increasing doses of zaragozic acid for 96 hours and concentrated CM was run by native-PAGE, followed by immunoblotting for ApoE. A duplicate gel of the same samples was run in tandem, and the gel was stained with Imperial Blue protein stain to visualize total protein. C, control; S, simvastatin; Z or Zara, zaragozic acid. Data in (A) collected by Dr. Josh Kulas.

I conducted follow-up zaragozic acid dose-response experiments using WT astrocyte-enriched cultures and ran native gels for ApoE lipoparticles to confirm this shift in lipoparticle size. Indeed, I was able to confirm Dr. Kulas's finding and show that the presence of the smaller ApoE band increased with higher concentrations of the drug (Figure 3.4.1B). Notably, in my experiments, I was able to see the lower MW band in the control samples as well, which varied from Dr. Kulas's original blots. Nonetheless, the significant upregulation of the smaller ApoE lipoparticles based on zaragozic acid dosage is readily apparent and consistent with Dr. Kulas's data. This was an intriguing result, but it could be argued that it may have been expected, as cholesterol is needed for ApoE lipoparticle generation. Since we are impairing cholesterol synthesis, we are likely reducing the cellular pool of cholesterol that can be added to synthesize the ApoE particles, thereby resulting in their smaller size. We wanted to assess whether this was the case or not.

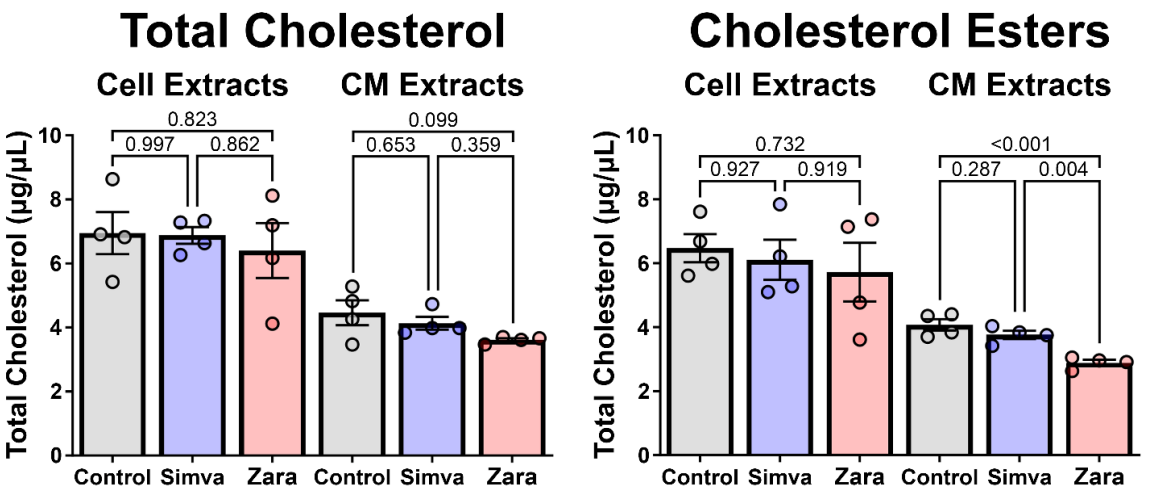


Figure 3.4.2. Zaragozic acid treatment reduces the amount of secreted cholesterol esters, not total cholesterol levels. Astrocyte-enriched glial cultures from WT mice were treated with either DMSO, 2µM simvastatin, or 1µM zaragozic acid for 96 hours. Cell lysate and conditioned media (CM) was collected and cholesterol was extracted from each sample. Amplex Red calorimetric assays were conducted in the presence or absence of exogenous cholesterol esterase to determine levels of total cholesterol and cholesterol esters, respectively. N=4 independent experiments/condition, one-way ANOVA within sample type. Detected cholesterol levels are graphed as mean ± s.e.m. Simva, simvastatin; Zara, zaragozic acid. Data collected by Dr. Josh Kulas.

We sought to determine whether simvastatin or zaragozic acid treatment altered levels of cellular and secreted cholesterol and cholesterol esters, as cholesterol levels are known to impact cholesterol efflux and uptake. We found that zaragozic acid alone significantly reduced levels of cholesterol esters in the conditioned media without altering cellular or secreted total cholesterol levels (Figure 3.4.2). Combined with our results from Supplementary Figure 2.1 showing that zaragozic acid increases transcripts of *Hmgcr* and *Srebf2*, as well as FDFT1 protein levels, our data suggests that specifically inhibiting cholesterol synthesis upregulates the mevalonate pathway while having only a small effect on esterified cholesterol levels, at least at the time point we assessed. This could be one explanation for why ApoE lipoparticles from cells treated with zaragozic acid are

significantly lower in size, and I believe that this is likely the case. Despite this, I also believe that this data implies that astrocytes predominantly utilize newly-synthesized cholesterol to generate ApoE lipoparticles. To my knowledge, this is not fully understood or characterized. However, there are multiple experiments that need to be conducted to address whether this is the case. These will be discussed in the following section, along with experiments related to ApoE isoform differences, lipoparticle composition, and modulating cholesterol biosynthesis for the treatment of AD.

3.4.2. Future directions

As a first step, I believe it would be highly beneficial to confirm the findings of our native gels through alternate methods. In particular, we can use fast protein liquid chromatography (FPLC) to separate out lipoparticles by size into different fractions. We could then run these fractions on WBs to identify the fractions that ApoE lipoparticles were eluted from the column in. Comparing the fractions that ApoE particles are eluted from in control- and zaragozic acid-treated cells would allow us to confirm our findings that zaragozic acid reduces ApoE lipoparticle size in our system. I did an initial run of this experiment with the help of Dr. Ira Schulman in the Department of Pharmacology at University of Virginia that yielded promising results after running the FPLC. We used a GE Superose 6 10/300 GL column and loaded 100µL of unconcentrated conditioned media from WT astrocyte-enriched glial cultures treated with control or zaragozic acid for 96 hours. With this column, larger, more massive particles are eluted earlier in the fractions, while lighter, smaller particles elute off in later fractions. In the control sample, we were able to see two peaks of protein eluting in fractions 22-33 that were absent in the zaragozic acid sample (Figure 3.4.3). This was exciting as it suggested that there was less protein in fractions that would contain larger particles after zaragozic acid

treatment, which would confirm our native gel data. However, I was unable to see any ApoE protein when I ran the eluted fractions on western blots. I attempted multiple methods for trying to see the ApoE in the eluted fractions by WB and was unsuccessful in doing so. The project then took a turn for another direction and I did not do many more attempts to optimize this procedure. If I were to do this experiment again, I would run ELISAs on the eluted fractions rather than WBs. This would allow us to run more samples at the same time, but more importantly, ELISAs are much more sensitive than WBs and may be more readily able to detect differences in highly diluted samples, as the eluted fractions are. This would allow us to corroborate our native gel data and be more confident that ApoE lipoparticle size is reduced in response to cholesterol synthesis inhibition with zaragozic acid.

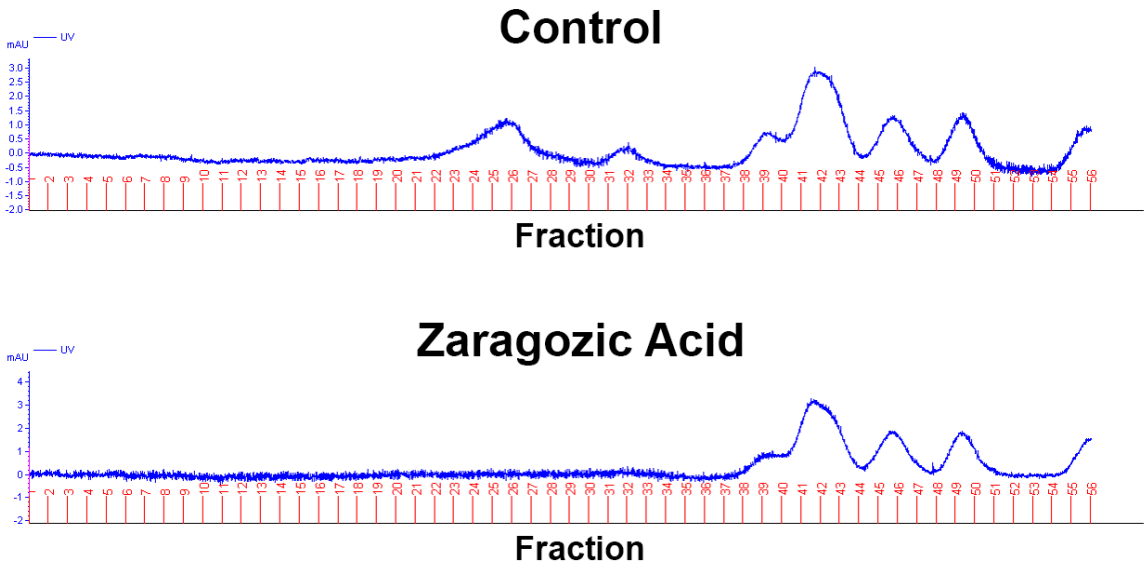


Figure 3.4.3. FPLC-based separation of particles by size after zaragozic acid treatment. Astrocyte-enriched mixed glial cultures from WT mice were treated with either DMSO or 1μM zaragozic acid for 96 hours. Conditioned media was collected and run on an FPLC column. Protein concentration was assessed during FPLC fractionation and is presented.

The next experiment that I think would yield strong and interesting data would be to assess my suspicion that astrocytes utilize newly-synthesized cholesterol to generate ApoE lipoparticles. To do so, I would collaborate with a lab capable of conducting radiolabeling experiments. For this experiment, I would spike cells pre-treated with DMSO as a control and 1μM zaragozic acid with [^{3}C]-D-glucose, which the cells would subsequently use and incorporate into cholesterol molecules[378]. After an optimized amount of incubation time, I would collect and concentrate the conditioned media, extract the lipids, and analyze levels of radiolabeled cholesterol by mass spectrometry (Figure 3.4.4A). This would allow us to track newly-synthesized cholesterol in the media, likely ApoE-associated, under our drug conditions to determine whether *de novo* synthesized cholesterol is used for ApoE lipoparticle generation, and whether zaragozic acid interferes with this.

While this experiment may yield interesting data, it is not without its downsides. First, this experiment would be difficult, as it may be challenging to get a high enough yield of cholesterol from the conditioned media to accurately measure radioactivity. Second, it would not tell us about incorporation of newly-synthesized cholesterol into ApoE lipoparticles specifically *per se*, as we would just be measuring the amount of newly-synthesized cholesterol that was secreted from the cells. To do so, we might have to immunoprecipitate out ApoE in the media and then measure radioactivity in the particles. While I am not by any means an expert in the lipid mass spectrometry field, I am positive based on my readings that this would be an extremely difficult thing to do and get true, reliable results. Nonetheless, these may be some experiments that could be conducted to get a better sense of whether newly-synthesized cholesterol is the predominant source of cholesterol in ApoE lipoparticles or not. We may also be able to

test this by conducting time-course experiments using zaragozic acid and other cholesterol synthesis inhibitors, as some have done previously[379], which would be a simpler, but less elegant way of testing this hypothesis.

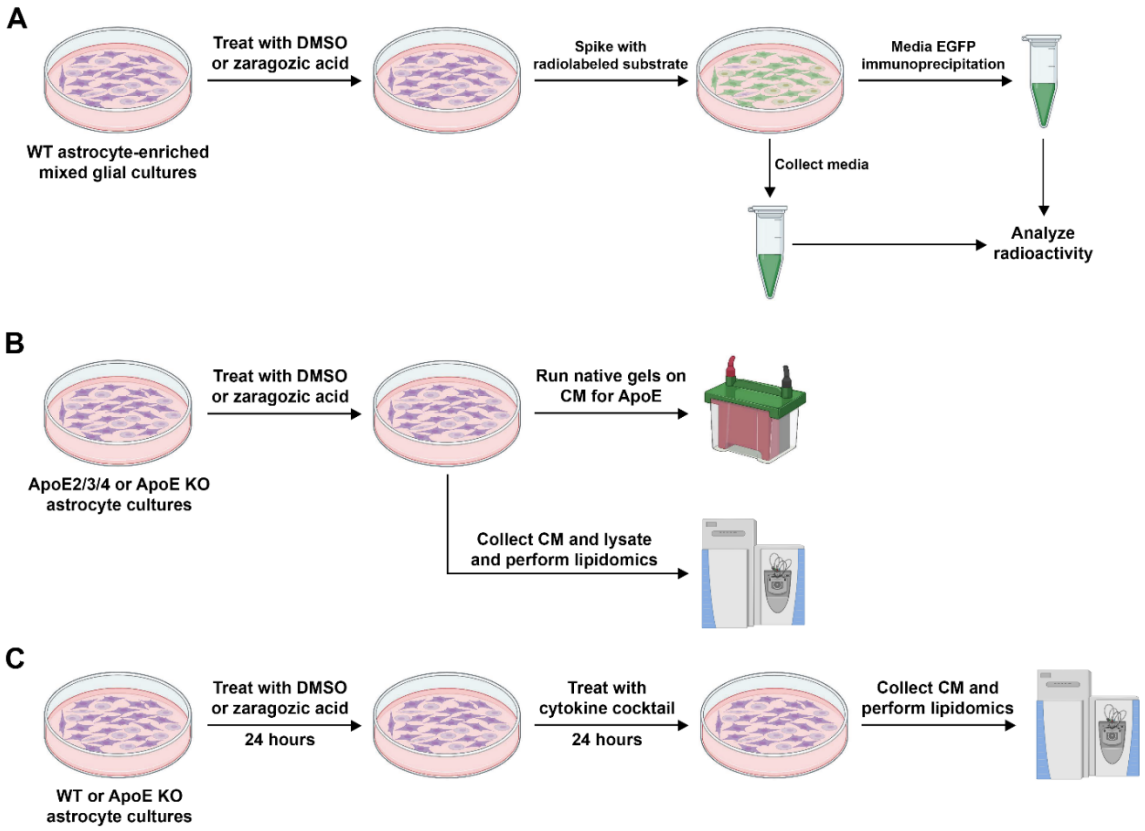


Figure 3.4.4. Treatment strategy for measuring ApoE lipidation *in vitro*. (A) Astrocyte-enriched mixed glial cultures from WT mice would be pre-treated with either DMSO or 1μM zaragozic acid for 96 hours. The cells would then be spiked with radiolabeled cholesterol substrate. The conditioned media from these cells would then undergo EGFP immunoprecipitation or not, followed by its subsequent analysis for radioactive cholesterol levels. **(B)** Astrocyte-enriched glial cultures from ApoE TR or ApoE KO mice would be treated with DMSO or 1μM zaragozic acid for 96 hours. The conditioned media (CM) would be concentrated and run on a native gel to compare ApoE lipoparticle size in response to zaragozic acid in all three genotypes. Alternatively, the CM and lysate from these treated cells would be collected and lipidomics would be performed. **(C)** WT and ApoE KO astrocyte-enriched mixed glial cultures will be treated with DMSO or 1μM zaragozic acid for 24 hours before being given a proinflammatory cytokine cocktail to induce activation. After 24 hours of activation and 48 hours of zaragozic acid treatment, conditioned media will be collected and analyzed by lipidomics analysis.

ApoE lipoparticles vary in size and composition based on isoform[66]. Given that there are differences in lipid metabolism in cells harboring different ApoE isoforms, it would be interesting to know whether zaragozic acid has a similar effect on ApoE KI glia as it did for glia expressing the endogenous mouse ApoE. To test this, I would conduct the same experiments as outlined in the preliminary data section with primary astrocyte-enriched mixed glial cultures from ApoE TR mice. Specifically, after treating the cells with DMSO or zaragozic acid for 96 hours, I would collect and concentrate the conditioned media, run the samples on a native gel, and immunoblot for ApoE to assess lipoparticle size (Figure 3.4.4B). My hypothesis would be that zaragozic acid would have the strongest effect on ApoE2 cells, followed by ApoE3 and then ApoE4 because it is more highly lipidated than the others, thus having more lipid to lose due to cholesterol synthesis inhibition.

As discussed, the lipid composition of astrocyte-derived ApoE lipoparticles can have strong impacts on the health of other brain cells[51]. As such, it would be of high

interest to characterize the lipid signature of zaragozic acid-treated cells compared to controls. To do so, I would treat astrocyte-enriched mixed glial cultures from ApoE TR mice, as well as ApoE KO mice with zaragozic acid or control for 96 hours, collect both the conditioned media and cell lysate, and conduct lipidomics analysis on each compartment in each condition. This may yield crucial results to understanding how cholesterol biosynthesis modulates secreted and intracellular lipid composition (Figure 3.4.4B). Comparing the results from the ApoE TR cells to the ApoE KO cells could give insight into the role ApoE plays in the secreted lipid profiles of cells in the presence of a cholesterol synthesis inhibitor, and how these differ by ApoE isoform.

Given that astrocytes secrete ApoE lipoparticles containing neurotoxic long-chain saturated fatty acids in response to inflammatory activation[51], it would also be a highly interesting experiment to determine whether cholesterol synthesis inhibition could mitigate the neurotoxic lipid profile of these ApoE particles after inflammatory activation. For this experiment, I would treat astrocyte-enriched mixed glial cultures from WT and ApoE KO mice with DMSO or zaragozic acid for 24 hours. I would then give the cells an established proinflammatory cytokine cocktail consisting of IL-1α, TNFα, and C1q for 24 hours[50]. After 24 hours of inflammatory activation and 48 hours of cholesterol synthesis inhibition, the conditioned media and cell lysate would be collected and sent to undergo lipidomics analysis (Figure 3.4.4C). Assessment of long-chain saturated fatty acid content in zaragozic acid-treated cells compared to controls may identify cholesterol biosynthesis inhibition as a potential therapeutic intervention for reducing the cytotoxicity of ApoE lipoparticles during the chronic neuroinflammation seen in a number of neurodegenerative diseases, including AD. Additionally, comparing WT to ApoE KO cells will allow us to determine the necessity of ApoE in this.

After determining whether zaragozic acid mitigates the amount of long-chain saturated fatty acids in astrocyte-derived ApoE lipoparticles, I would propose an experiment to determine the impact of cholesterol biosynthesis inhibition on AD pathology. Others have shown that modulation of other aspects of cholesterol and lipid metabolism may have therapeutic potential[285–287,290]. Therefore, it would be of interest to determine whether long-term administration of a cholesterol synthesis inhibitor in a mouse model of AD would alleviate generation of pathology. While some studies have used zaragozic acid to inhibit cholesterol synthesis in the liver of mice[380], its blood-brain-barrier (BBB) permeability is not well-characterized. In addition, it is known that due to zaragozic acid's target being squalene synthase, treating cells *in vitro* with the drug leads to an approximately 250-fold increase in FPP levels and a 4-fold increase in GGPP levels[381,382]. As such, for treating AD mice *in vivo*, I would use another cholesterol synthesis inhibitor such as AY9944, which inhibits 7-dehydrocholesterol reductase (DHCR7), the final step in the cholesterol biosynthesis pathway. This drug is known to cross the blood brain barrier and reduce cholesterol production in the brain within 3 days when a constant drug injection using osmotic pumps implanted under the dorsal skin of mice is used[379]. To assess the effect of this drug on AD pathology, I would use the amyloidogenic 5xFAD model of AD, as well as the PS19 model of tauopathy, as these are more aggressive models that lead to early development of AD pathology by the time the mice are about 3-6 months of age. So as not to inhibit peripheral cholesterol synthesis, as this may have deleterious effects, I would treat control and AD mice with AY9944 via intracerebroventricular (ICV) injection using an osmotic pump beginning around 3 months of age, the approximate age of disease onset. As AY9944 is a potent inhibitor of cholesterol synthesis, it is normally only used for 3 days of continuous injection. As such, I would optimize a lower dose of the drug such that we could

efficiently inhibit cholesterol synthesis without confounding effects of long-term treatment. I would inject the mice for 1 month with either AY9944 or a control, collect the brains of the mice after the treatment period, and analyze levels of either $A\beta_{1-40}$ and $A\beta_{1-42}$ or phosphorylated tau depending on the model. I would also stain amyloid plaques and tau tangles using immunofluorescence techniques to visualize AD pathology changes in the brains of these mice.

Although this experiment would allow us to determine whether altering cholesterol synthesis may impact the generation of AD pathology, it is not without its downsides. It may be the case that long-term inhibition of cholesterol synthesis leads to unforeseen negative effects, and that we are unable to inject for a long-enough time to be able to observe an impact on amyloid or phosphorylated tau production. In this case, an alternative method would be to conduct *in vitro* experiments using primary cultures of astrocyte-enriched glia, as well as neurons from these AD mouse models. I would culture primary astrocyte-enriched mixed glial cultures from these mice and treat these cells with the cholesterol synthesis inhibitors for 96 hours. I would then collect the conditioned media from the treated astrocyte-enriched cultures, and incubate this conditioned media on the neuron cultures from the AD model mice. As we have previously shown that this experimental setup is able to modulate Aβ production in neurons based on ApoE lipidation status[317], this is an effective and more direct way to determine whether cholesterol synthesis inhibition in astrocytes modulates ApoE lipoparticle composition and subsequently Aβ production in neurons. Conducting the same experiment in cultures derived from a mouse line where AD mice have been crossed with ApoE KO mice can also give information of whether this potential effect on amyloid production is dependent on astrocyte-derived ApoE lipoparticles.

3.5. Conclusion

ApoE and the mevalonate pathway are critically involved in regulating normal brain homeostasis, and also contribute to the pathogenesis and progression of AD. Understanding the interplay between ApoE, prenylation, and cholesterol is essential for identifying how these processes contribute to AD pathology. The experiments outlined in this chapter may shed light on some of these questions, allowing for a better characterization of the regulatory mechanisms underlying ApoE lipoparticle generation, secretion, uptake, and recycling. This is imperative for identifying novel targets for therapeutic interventions targeting ApoE in order to treat AD.

Appendix 1. The brain as an insulin-sensitive metabolic organ

A1.1. ABSTRACT

A1.1.1. Background:

The brain was once thought of as an insulin-insensitive organ. We now know that the insulin receptor is present throughout the brain and serves important functions in whole-body metabolism and brain function. Brain insulin signaling is involved not only in brain homeostatic processes but also neuropathological processes such as cognitive decline and Alzheimer's disease.

A1.1.2. Scope of review:

In this review, we provide an overview of insulin signaling within the brain and the metabolic impact of brain insulin resistance and discuss Alzheimer's disease, one of the neurologic diseases most closely associated with brain insulin resistance.

A1.1.3. Major conclusions:

While brain insulin signaling plays only a small role in central nervous system glucose regulation, it has a significant impact on the brain's metabolic health. Normal insulin signaling is important for mitochondrial functioning and normal food intake. Brain insulin resistance contributes to obesity and may also play an important role in neurodegeneration.

A1.2. INTRODUCTION

With the introduction of insulin in 1921, diabetes, then a near uniformly fatal disease, became manageable. But along with that initial increase in lifespan, many of the complications of diabetes became apparent. Early research focused on the organs

most associated with morbidity and mortality, including the kidney and heart, as well as the classically insulin-sensitive tissues responsible for much of glucose homeostasis, such as liver and skeletal muscle. As this research has led to increasingly longer lifespans for those affected by diabetes, it has become apparent that no organ, including the brain, is spared by the disease. The huge advances in molecular biological and neuroscience tools over the past 30 years have opened the way for greater understanding of how the brain, a non-classical insulin-sensitive tissue, is impacted by diabetes. Intriguingly, the potential roles for insulin in the pathogenesis and treatment of some neurological diseases, including depression, cognitive decline, and Alzheimer's disease (AD), have expanded the field of brain insulin signaling research beyond the confines of diabetes.

While the brain comprises only 2% of the human body's overall mass, it utilizes an estimated 20% of the body's glucose. For the most part, this glucose utilization is not dependent on insulin-stimulated translocation of the glucose transporter 4 (GLUT4), as is seen in classical insulin-sensitive tissues like adipose tissue and skeletal muscle. As such, the brain was labeled early on as an insulin-insensitive organ, whose glucose utilization was mediated through insulin-independent mechanisms. Since that designation, a vast amount of research has shown that the brain is, in fact, insulin-sensitive, despite its ability to uptake glucose being, for the most part, insulin-independent. The first hints at the brain's insulin sensitivity came from studies showing widespread expression of the insulin receptor (IR) in the brain. Since then, we have learned that the brain regions that harbor high levels of IR expression are some of those most known for their roles in cognition and feeding behaviors. In addition, it is now apparent that insulin and its close relative, insulin-like growth factor 1 (IGF1), are both

able to influence brain metabolism and cellular function. This review aims to present what is currently understood about the role of insulin in the brain, beginning with its transport across the blood-brain-barrier (BBB) and its signaling effects on cellular function and metabolism. We will also discuss insulin's role in modulating the activity of different brain circuits and the resulting behavioral and metabolic implications arising from this. Finally, we will address cognitive impairment and Alzheimer's disease, which are associated with insulin resistance, and the potential for combatting them with intranasal insulin and insulin sensitizers.

A1.3. INSULIN RECEPTOR EXPRESSION AND SIGNALING IN THE BRAIN

A1.3.1. Brain insulin receptor expression

As noted above, a critical first step in recognizing the brain as an insulin-sensitive organ was the identification of IR expression in a variety of brain regions. Although the insulin receptor is found ubiquitously throughout the brain, its expression is at higher levels in select regions, such as the cerebellum, cortex, and hypothalamus[383]. The receptor itself is primarily found in the plasma membrane and is composed of dimers of α and β subunits. It is important to note that neurons and glial cells, such as astrocytes, express different isoforms of the α subunit of the insulin receptor. Neurons express the IR-A isoform, which excludes exon 11, whereas glia predominantly express IR-B, which includes exon 11[384–386]. This differs from peripheral tissues where the majority of IRα expression is the IR-B isoform. Also in contrast to the brain, IRα isoform expression patterns in the periphery are largely tissue-dependent, rather than cell-type-dependent[387]. Despite this, there has not been evidence to suggest differing affinities for insulin between central and peripheral IRs. In general, studies assessing the affinities for

the IR-A and IR-B isoforms for insulin have concluded that the IR-A isoform has approximately a 1-2 fold higher affinity compared to the IR-B isoform (EC_{50} calculations range from 0.4-6nM depending on the study and methods used)[388–393].

To add even more complexity to the receptor-ligand interactions, heterodimers consisting of an IR and a receptor for IGF1 (IGF1R) have been described in the brain and in the periphery[386,394] (Figure A1.1). As a result, there are five possible combinations of receptor dimerization between the IR and IGF1R, including the homodimers IR-A:IR-A, IR-B:IR-B, and IGF1R:IGF1R, as well as the heterodimers IR-A:IGF1R and IR-B:IGF1R. These heterodimers, also referred to as hybrid receptors, seem to have increased affinity for IGF1 compared to insulin, although they are able to bind both of these hormones, along with IGF2, at varying affinities. IR-A:IGF1R and IR-B:IGF1R hybrid receptor EC_{50} values for insulin are widely variable between studies (~1-350nM for IR-A:IGF1R and ~1-325nM for IR-B:IGF1R), with some showing no difference in insulin binding and some showing higher affinity by the IR-A:IGF1R hybrid receptor[390,393,395–398]. Thus, more work is required to better clarify or confirm the different substrate binding efficiencies of these two hybrid receptors. The extent to which IRs and IGF1Rs are in a heterodimer or homodimer conformation is believed to be dependent on the number of receptors in the tissue, as the energy required for making either a hetero- or homodimer is equivalent[387,396,398]. How these heterodimers and homodimers differentially impact the brain's response to insulin requires additional research, although some work has been done to elucidate this *in vitro*. Specifically, Cai et al.[384] reported that the primary contributing factor to the downstream signaling effects of the IR and IGF1R is the intracellular juxtamembrane domains of these two receptors, not the extracellular domain or substrate. While this study gives insight into what contributes to the

differential signaling by IR and IGF1R homodimers after activation, the downstream effects of IR/IGF1R hybrid receptor signaling has not been well-characterized thus far.

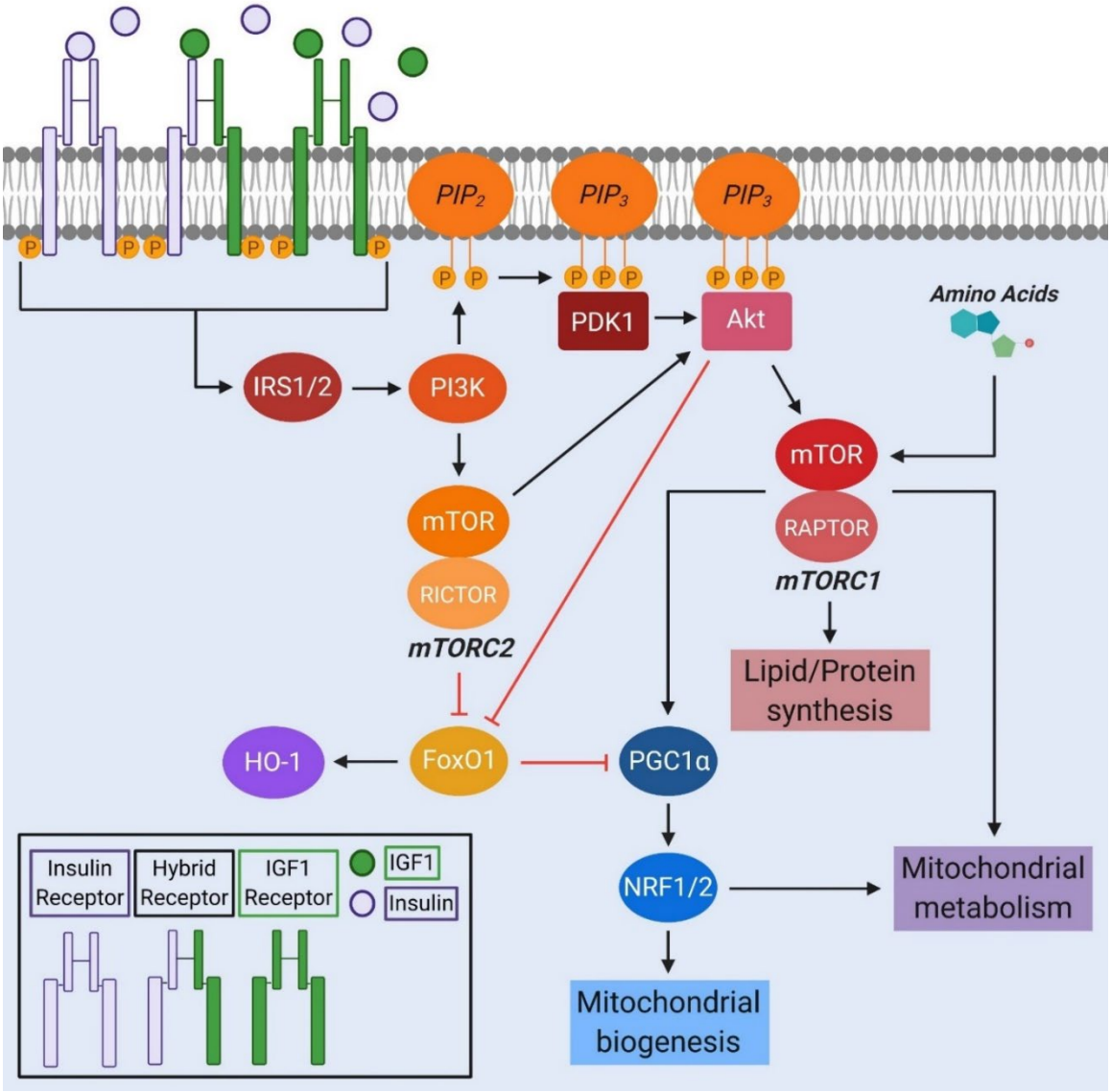


Figure A1.1. Insulin signaling and mitochondria. Binding of insulin and IGF1 to IR and IGF1R homo- and heterodimers (also known as hybrid receptors) initiates a signaling cascade that activates IRS1/2 and PI3K, which in turn activates mTORC2 and Akt. Both mTORC2 and Akt inhibit FoxO1 to prevent transcription of HO-1. Activation of mTORC1 by Akt and amino acids promotes lipid and protein synthesis, as well as mitochondrial metabolism and biogenesis through the PGC1α-NRF1/2 pathway. Akt-protein kinase B, FOXO1-forkhead box O1, HO-1-heme oxygenase-1, IGF1-insulin-like growth factor 1, IRS1/2-insulin receptor substrate 1/2, mTORC1/2-mammalian target of rapamycin complex I/2, NRF1/2-nuclear respiratory factor 1/2, PGC1α-peroxisome-proliferator activated receptor coactivator-1α, PI3K-phosphatidylinositol 3-kinase.

A1.3.2. Insulin production and transport across the BBB

The question as to whether the brain produces its own insulin or if it is all or at least mostly pancreatic in origin has been controversial for decades. Pancreatic preproinsulin is processed in the endoplasmic reticulum and requires the expression and activity of multiple endopeptidases in order to cleave the C-peptide fragment from proinsulin. This generates the mature form of insulin that will subsequently be exocytosed in secretory granules by β-cells in response to elevated blood glucose levels. Humans and rabbits have a single gene encoding for insulin, while rodents have two. Of the two *Ins* genes that lead to the production of preproinsulin in rodents, *Ins II* appears to be the one predominantly expressed by neurons[399,400]. In cultured rabbit neurons and glia, only neurons are able to secrete insulin into the culture media[400]. While *Ins II* may be expressed in the brain *in vivo*, whether the endopeptidases that are required for secretion of the mature hormone product are expressed in the brain has been less well-demonstrated. Overall, data suggesting that the human brain makes and secretes significant amounts of insulin locally is lacking and it is largely believed that the vast majority of insulin found in the brain parenchyma originates from the pancreas. To

allow for this, transport of insulin from the blood into the brain must occur via passage through the BBB and/or the blood-cerebrospinal fluid (CSF) barrier.

The concentration of insulin in the CSF is significantly lower than that found in the blood. Multiple studies have shown that the concentration of CSF insulin increases much slower and peaks at much lower levels than that of plasma insulin levels during hyperinsulinemic-euglycemic clamps[388,401–403]. Together with the fact that insulin is a 51-amino acid peptide, these findings suggest that insulin's transport into the brain is not through diffusion and instead requires active mechanisms. These mechanisms have not been fully elucidated, but studies using radiolabeled insulin have found that insulin quickly localizes to the brain endothelial cells, which can uptake the insulin in an IR-dependent manner[388,404–406]. Confirming a role for endothelial IR in insulin transport across the BBB, endothelial cell-specific IR knock-out (IRKO) mice injected with insulin intravenously have reduced downstream insulin signaling in the hippocampus, hypothalamus, and frontal cortex[407]. Insulin can also be transported across circumventricular organs, such as the median eminence, where the vessels of the BBB are fenestrated. Here, insulin may be brought into the CSF or interact with specialized ependymal cells called tanycytes that facilitate receptor-mediated endocytosis of insulin for transport to neurons in the hypothalamus[388,408].

Notably, there is controversy in regards to the measurement of CSF insulin concentrations as a means of extrapolating brain parenchymal insulin levels. CSF insulin has been used as a proxy for tissue insulin given the relative ease of collection, however, insulin levels in the CSF are very low and may not reach levels needed to induce insulin signaling in the brain[388,401,402]. Complications with measuring brain insulin levels directly via microdialysis make interpreting these data difficult. An in depth

discussion of insulin transport and confounds of insulin concentration measurements between compartments can be found in a recent review by Gray and Barrett[388].

A1.3.3. Insulin signaling and glucose uptake in the brain

Once it has entered the brain, insulin binds to its receptor and initiates a series of phosphorylation events. First, an autophosphorylation event occurs on the intracellular tail of the IR, thereby recruiting the insulin receptor substrates 1 and 2 (IRS1 and IRS2). Tyrosine phosphorylation of IRS1/2 leads to downstream activation of the kinases phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt), and mammalian target of rapamycin (mTOR)(Figure A1.1)[409,410]. This PI3K/Akt/mTOR signaling pathway impacts a wide variety of cellular functions, including synaptic plasticity, cholesterol synthesis, neuronal survival, and trafficking of neurotransmitters[411–414]. Insulin is also able to modulate cell growth and proliferation through induction of Shc and its downstream targets Ras, ERK, and mitogen-activated protein kinase (MAPK). Although insulin can promote this pathway, it appears to be more active in response to IGF1R signaling compared to IR signaling[384]. In addition, some evidence suggests that the different IRα isoforms promote different downstream signaling, with the IR-A isoform preferentially activating the mitogenic Shc/Ras/ERK/MAPK pathway and the IR-B isoform activating the PI3K/Akt/mTOR pathway[386,398].

The primary difference between peripheral and brain insulin signaling is the regulation of glucose transporters. In peripheral tissues, insulin-mediated Akt activation induces translocation of glucose transporter 4 (GLUT4) from vesicles to the plasma membrane to facilitate glucose uptake from the blood[415]. In the brain however, the expression of GLUT4 is limited to specific brain regions, such as the hippocampus and hypothalamus, resulting in a much smaller impact on glucose uptake[416,417]. Thus, it is

primarily the glucose uptake effects of insulin signaling, rather than the signaling pathways themselves, which differ between the brain and periphery.

In the brain, glucose import from the circulation is primarily mediated through the insulin-insensitive GLUT1, which is expressed by endothelial cells and astrocytes at the BBB[418–420]. Within the brain parenchyma, GLUT3 and GLUT1, both of which are considered insulin-insensitive, are expressed widely by neurons and glial cells, respectively. There is evidence to support some function of insulin in regulating brain glucose uptake. Astrocyte-specific IRKO in adult mice results in decreased CSF glucose levels after peripheral glucose injection[421]. Furthermore, *in vivo* ^{18}FDG-PET imaging of astrocyte-specific IRKO mice shows decreased glucose uptake in the brain and this effect coincides with diminished GLUT-1 mRNA expression[421]. There is also evidence that insulin can indirectly induce GLUT3 translocation to the plasma membrane in neurons to allow for glucose uptake[422], suggesting that insulin does play a role in modulating glucose transporter expression in the brain, despite the limited expression of insulin-sensitive GLUTs. Furthermore, Fernandez et al.[423] identified a putative mechanism by which astrocytes can uptake glucose via GLUT1 translocation in response to concurrent signals from insulin and IGF-1. Together, these data indicate that insulin signaling may play an important role in regulating glucose uptake into the brain through non-traditional pathways.

Despite these findings, the majority of glucose uptake by the brain is not regulated by insulin signaling. While this holds true for brain glucose metabolism as we currently understand it, insulin does play a critical role in modulating metabolic activity intracellularly through its regulation of metabolic signaling pathways.

A1.3.4. Control of cellular metabolism by insulin

Diseases related to impaired insulin signaling, commonly referred to as insulin resistance, are predominantly associated with altered metabolic function. As such, it is not surprising that insulin affects many aspects of cellular and mitochondrial metabolism, not only in the periphery, but also in the brain. As the primary producers of cellular ATP, mitochondria are critical in maintaining metabolic homeostasis, and mitochondrial dysfunction is observed in many metabolic diseases that are characterized by insulin resistance.

As mentioned, the insulin signaling cascade is associated with activation of Akt, which leads to assembly of mTORC1. mTORC1 signaling is critical for regulating protein, lipid, and fatty acid synthesis, as well as mitochondrial metabolism[424–427]. In terms of its metabolic impact, mTORC1 is integral to mitochondrial oxidative metabolism and biogenesis through its control of peroxisome-proliferator activated receptor coactivator (PGC)-1α and the transcription factors nuclear respiratory factors 1 and 2 (NRF1/2) (Figure A1.1)[428,429]. In addition to activation by insulin through Akt, mTORC1 can be activated by increased levels of amino acids[430–432]. When mTORC1 is activated by either insulin or amino acids, production of nuclear-encoded mitochondrial proteins is stimulated. These are then incorporated into a variety of mitochondrial metabolic pathways, including the tricarboxylic acid (TCA) cycle, fatty acid β-oxidation (FAO), and the electron transport chain complexes[433]. Further, mTORC1 drives a metabolic shift from oxidative phosphorylation (OXPHOS) to glycolysis during cell growth and development. The ability for mTORC1 to sense amino acids and regulate both mitochondrial metabolism and biogenesis places it as a central player controlling cellular metabolism and nutrient sensing. This may be important when it comes to the regulation

of metabolism in different brain cell types, both under normal conditions and under metabolic stress. For example, mTOR-driven autophagy in response to metabolic stress appears to be more robust in astrocytes compared to neurons[434]. Therefore, it is possible that regulation of metabolism by mTORC1 signaling may be different between these cell types. Given that astrocytes utilize glycolysis and FAO as their primary means of energy production while neurons preferentially use OXPHOS[435–438], it may be that differences in mTOR signaling play a role in establishing these metabolic phenotypes. However, this comparison and whether the variable mTOR signaling in these cells differentially affects their mitochondrial response to nutrient deprivation have yet to be addressed.

mTOR is also found in a second complex, mTORC2, which acts through Akt to promote cell proliferation and survival. mTORC2-mediated Akt activation also negatively regulates forkhead box O1 (FoxO1) (Figure A1.1). FoxO1 promotes the transcription of heme oxygenase-1 (HO-1). Excess hepatic HO-1 expression in the liver impairs mitochondrial OXPHOS and FAO[439] by decreasing mitochondrial biogenesis[439,440]. Therefore, insulin's activation of Akt inhibits FoxO1-dependent HO-1 transcription and prevents HO-1 hyperactivation-induced mitochondrial dysfunction in the liver. Whether this same process occurs in the brain has not been determined. However, there is evidence suggesting an important role of insulin-mediated regulation of FoxO1 in controlling food intake, insulin sensitivity, and glucose homeostasis. Mice with constitutively-active, nuclear FoxO1 have elevated food intake and associated obesity, while mice with deletion of FoxO1 in hypothalamic neurons have diminished food intake[441–446].

Insulin's other major signaling pathway, which involves activation of Ras/ERK/MAPK, also modulates mitochondrial homeostasis and function. In *in vitro* experiments using a hypothalamic neuronal cell line, Wardelmann et al.[447] found that insulin acts through ERK to induce the expression of the mitochondrial chaperones heat shock protein (Hsp) 60 and Hsp10. They further identify this pathway as a potential mediator of insulin-induced mitochondrial respiration, as inhibition of ERK signaling alleviated the induction of mitochondrial respiration due to insulin treatment. It should be noted that they found the same response with IGF1 acting through the IGF1R. Whether *in vivo* these effects are primarily driven by IGF1R signaling or IR signaling remains to be determined. Altogether, brain insulin action has clear roles in regulating cellular metabolism despite its limited impact on modulating glucose uptake into the brain.

A1.4. BRAIN CIRCUITRY AND BEHAVIOR IN RESPONSE TO INSULIN

A1.4.1. Insulin signaling in neuronal populations

As touched upon above, one of the prominent effects that insulin exerts on the brain is regulation of feeding behaviors. This is done, in part, through its binding to IRs on pro-opiomelanocortin (POMC) and agouti-related protein (AgRP) neurons in the arcuate nucleus of the hypothalamus. AgRP neurons are orexigenic and promote hunger while POMC neurons are anorexigenic and promote satiety. Thus, the individual activities of these neuronal populations oppose each other to modulate hunger and food intake. On balance, intracerebroventricular infusion of insulin reduces food intake in both fasted and non-fasted rodents and results in weight loss[388,448–451]. However, the role of insulin on these subpopulations of neurons extends beyond food intake and into the periphery through regulation of peripheral metabolism.

For example, central insulin resistance alters glucose sensing in hypothalamic neurons, leading to an impaired sympathetic outflow in response to hypoglycemia[452,453]. Insulin control of sympathetic outflow also seems to participate in body temperature regulation. When IR is knocked out from the brain, mice become hypothermic[454]. In contrast, intranasal delivery of insulin in humans promotes thermogenesis[455]. At least in rodents, this effect is mediated by sympathetic activation of brown fat[456].

Further evidence delineating the impact insulin signaling in hypothalamic neurons has on peripheral metabolism comes from cell-specific IRKO experiments in AgRP and POMC neurons. These studies have demonstrated that insulin signaling in AgRP neurons regulates hepatic glucose production, while insulin signaling in POMC neurons affects adipose tissue lipolysis (Figure A1.2)[457,458]. In support of this, insulin injection directly into the CNS increases insulin sensitivity in the liver, while also stimulating lipogenesis and accumulation of fat[459,460]. However, the part that insulin plays in POMC neuronal control of hepatic glucose production has been controversial, due to dichotomous results showing insulin can either inhibit POMC neuron activity[458,461–463] or promote it[464–466]. Although more work is needed to better understand these findings, a series of recent studies from the Tiganis group[467–469] have shed some light on this issue with the identification of T-cell protein tyrosine phosphatase (TCPTP), whose expression is increased during fasting and decreased post-prandially. This phosphatase appears to be able to determine whether insulin is inhibitory or excitatory to POMC neuronal firing and control of hepatic glucose production. Others have shown through single-cell profiling that POMC and AgRP neuronal populations in the hypothalamus are heterogeneous and these subsets may act differently in response to nutrient availability[470–472], further suggesting that the regulation of these neuronal populations

and their effects in the brain and periphery are much more complex than currently understood.

This complexity is likely reflected in human clinical trials of intranasal insulin. This delivery method, which allows for direct delivery of insulin to the brain, thus avoiding the potential for hypoglycemia, results in suppressed food intake in most trial participants[473,474]. However, one study which treated patients with intranasal insulin for 8 weeks found that while men lost body fat with the treatment, women did not[475]. This may represent a sex-based difference in the balance of hypothalamic control by centrally acting insulin of food intake versus stimulation of peripheral lipogenesis. Altogether, these various studies demonstrate that in addition to the important role insulin serves in regulating brain metabolism, there are multiple peripheral homeostatic functions that are fine-tuned by insulin signaling in the brain.

Convincing evidence supporting a specific role for insulin signaling in brain in cognitive or affective behaviors has been scarce until more recently. The commonly cited and used nestin-Cre IRKO (NIRKO) mouse does show some anxiety behaviors later in life[476,477]. This mouse is sometimes mistakenly referred to as a neuron-specific knockout, but nestin is an intermediate filament expressed during development by all neural progenitor cells. As such, the NIRKO mouse is a model of whole-brain IRKO (excluding microglia) rather than a neuron-specific deletion. Additional evidence implicates IR/IGF1R signaling in both the hippocampus and amygdala in behavior. Using adeno-associated virus (AAV)-Cre injection into both of these brain regions, Soto et al.[478] found that IR/IGF1R double-KO (DKO) in either brain region elevated anxiety behaviors and impaired systemic glucose homeostasis compared to mice injected with a control AAV. Further, hippocampal IR/IGF1R DKO mice had impaired spatial memory, whereas DKO

in the amygdala dysregulated brown adipose tissue thermogenesis[478]. These findings are consistent with a previous report that showed injection into the hippocampus of a lentiviral construct expressing an IR antisense sequence to downregulate IR expression specifically in this region negatively affected long-term potentiation and spatial memory[479]. While these two methods may have been able to decrease IR expression in the hippocampus and amygdala, they were not able to distinguish cell-type-specific effects of IR or IR/IGF1R DKO in these brain regions. Additionally, it will be critical to determine the individual contributions of insulin and IGF1 in long-term potentiation and spatial memory formation in the hippocampus and whether IR/IGF1R homodimers and heterodimers have distinct roles in these processes.

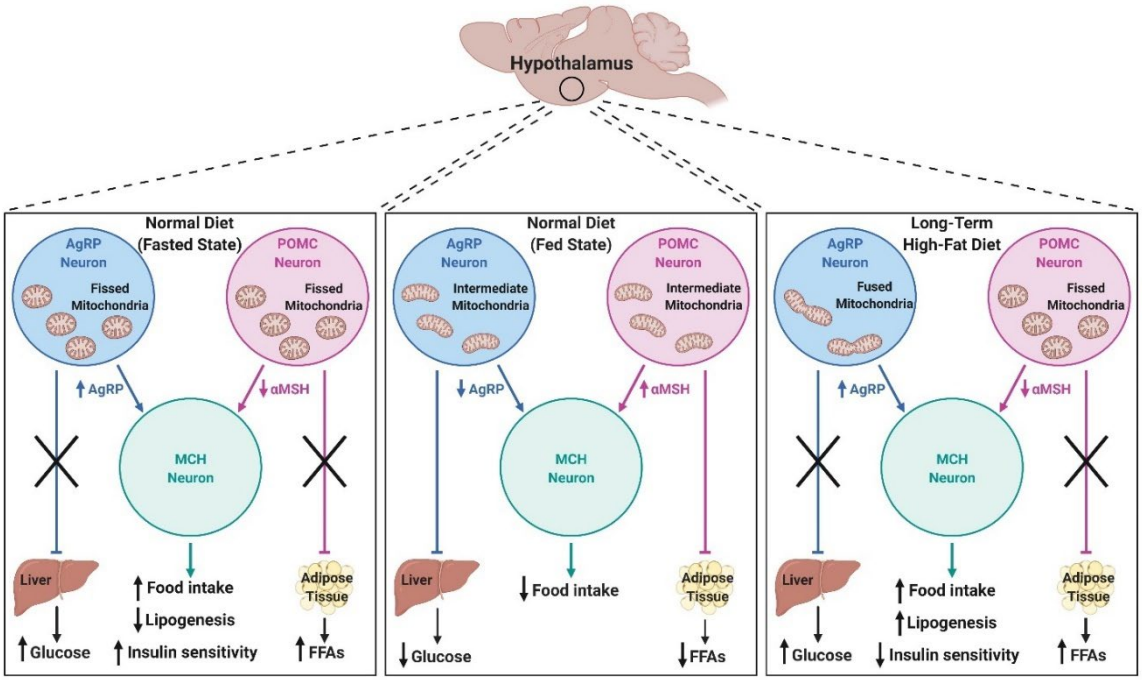


Figure A1.2. Hypothalamic regulation of whole-body metabolism under different feeding states. Fasting induces mitochondrial fission in both AgRP and POMC neurons of the arcuate nucleus of the hypothalamus. AgRP neurons produce more AgRP protein, while POMC-derived αMSH is decreased. These combined effects decrease the activity of MCH neurons in the paraventricular nucleus of the hypothalamus to increase food intake. AgRP and POMC regulation of hepatic glucose production and adipose tissue lipolysis is diminished to allow for elevated circulating levels of blood glucose and FFAs, resulting in reduced fat mass and higher insulin sensitivity. Under fed conditions, mitochondria in AgRP and POMC neurons are in an intermediate state, with a balance between mitochondrial fusion and fission. AgRP release is diminished and αMSH is enhanced, promoting MCH neuronal activity and satiety. IR signaling in AgRP and POMC neurons inhibits hepatic glucose production and lipolysis, respectively. In response to long-term HFD feeding, AgRP neuronal mitochondria enter a fused state, whereas mitochondria in POMC neurons are fissed. AgRP neurons upregulate production of AgRP protein, while αMSH production by POMC neurons is impaired, thereby inhibiting MCH neurons and increasing food intake. Impaired IR action on AgRP and POMC neurons leads to deficits in the ability of these neurons to inhibit hepatic glucose production and lipolysis, resulting in elevated fat mass and diminished insulin sensitivity. AgRP-agouti-related protein, FFAs-free fatty acids, MCH-melanin-concentrating hormone, αMSH-α-melanocyte stimulating hormone, POMC-pro-opiomelanocortin.

A1.4.2. Mitochondrial dynamics regulate hypothalamic neuron activity

Mitochondrial dynamics, among other aspects of mitochondrial biology, include fission and fusion of mitochondria and these processes are imperative for mitochondrial quality control and adaptation to the redox and energetic state of the cell. As the names imply, mitochondrial fusion is the process in which two discrete mitochondria combine into a single mitochondrion or when a single mitochondrion integrates into a mitochondrial network. In contrast, mitochondrial fission describes the separation of a single mitochondrion into two discrete organelles or the removal of a mitochondrion from the mitochondrial network. Mitochondrial fusion is primarily mediated by the proteins mitofusin 1 (MFN1), MFN2, and Opa1, whereas fission is mediated by the proteins dynamin-related protein 1 (Drp1), mitochondrial fission factor (MFF), and Fis1. Generally, when the cell is under energetic stress or nutrient deprivation, mitochondria will fuse and become elongated in an attempt to maximize energy production[480,481]. However, this response to stress may not be sustainable, as the increase in OXPHOS from these mitochondria may also augment ROS production and eventual mitochondrial damage[482]. Mitochondrial fission aids mitochondrial quality control by ensuring that old or damaged mitochondria are sequestered and isolated off of the mitochondrial network to be degraded through selective autophagy, termed mitophagy. In this way, fission is able to respond to increases in ROS or decreases in ATP production in order to mitigate these and limit downstream damage. These basic aspects of mitochondrial biology and homeostasis have been identified as imperative for the maintenance of mitochondrial adaptations to nutrient availability and neuronal activity in both AgRP and POMC neurons[40–43], which has direct implications for the regulation of whole-body insulin sensitivity and glucose homeostasis.

Under fasted conditions, mitochondria in both AgRP and POMC neuron populations undergo increased levels of fission, which coincides with decreased POMC activity and increased AgRP activity[40,42,43]. After feeding, mitochondrial fusion is upregulated in these cells, resulting in an intermediate phenotype of the mitochondrial network that is associated with diminished AgRP activation and enhanced POMC activation[40–43]. It is clear that the regulation of mitochondrial fission and fusion is required to allow for proper activation of POMC neurons under fed conditions. This comes from mouse studies demonstrating that POMC-specific KO of the fission regulator Drp1 or either of the fusion regulators MFN1/2 leads to altered POMC activity and impaired food intake and whole-body glucose tolerance[41–43](Figure A1.2). Mitochondrial fusion in POMC neurons may also be involved in the control of pancreatic glucose-stimulated insulin secretion by these cells, as POMC-specific MFN1 KO increased sympathetic outflow to the pancreas, resulting in reduced insulin secretion[43]. Taken together, mitochondrial dynamics in both AgRP and POMC neurons are not only affected by nutrient availability, but are also themselves important for these hypothalamic neuronal populations to function regularly and therefore, to modulate feeding behaviors, insulin sensitivity, glucose homeostasis, and fat storage throughout the body (Figure A1.2).

A1.4.3. Astrocyte insulin signaling

Astrocytes are integral for BBB integrity and neuronal metabolic and redox homeostasis[483]. It has become increasingly apparent that insulin signaling in astrocytes is imperative for these processes and also for modulating both behavior and whole-body glucose homeostasis[421,484]. Whereas NIRKO mice do not have any behavioral abnormalities until later in life, mice with astrocyte-specific IRKO exhibit depressive and anxiety behavioral phenotypes at an earlier age[484]. These effects have been attributed to

impaired dopamine release and signaling in the nucleus accumbens[484]. Further, these mice are hyperphagic and have impaired peripheral glucose tolerance, insulin sensitivity, and POMC neuronal firing in response to glucose[421]. IRKO from astrocytes in the hypothalamus showed similar whole-body effects, suggesting insulin signaling in hypothalamic astrocytes may serve a crucial role in modulating food intake and glucose homeostasis[421].

Interestingly, astrocyte IGF1 signaling also seems to be involved in learning and memory, as KO of IGF1R from astrocytes in mice impairs working memory[485]. As discussed, cultured astrocytes respond to both insulin and IGF1 by increasing GLUT1 expression and glucose uptake[423]. Individual roles for insulin and IGF1 signaling in modulating astrocyte metabolic activity have also been described in *in vitro* systems. IGF1R KO in primary astrocyte cultures reduces their basal oxygen consumption rate and adenylate energy charge[485], a measure of the energetic state of the cell that accounts for the levels of cellular AMP, ADP, and ATP. Similar to cultured neurons, treating primary cortical astrocytes with insulin suppresses H_2O_2 production and increases mitochondrial ATP production, suggesting that insulin-stimulated mitochondrial respiration occurs in both of these cell types[486]. IRKO astrocytes *in vitro* have diminished glycolytic activity, as evidenced by decreased glucose uptake and L-lactate release, and this appears to be due to a switch in their metabolic phenotype from glycolysis to FAO[421]. Because astrocytes contribute to neuronal metabolic homeostasis and respond to neuronal firing through the release of lactate[438,483,487], these findings may have implications for astrocyte insulin signaling in maintaining neuronal metabolism, as well as brain metabolic defects observed in insulin-resistant conditions. While this phenomenon appears to be present in humans as well, based on modeling of nuclear

magnetic resonance imaging data[488], whether this astrocyte metabolic phenotype switch is altered *in vivo* during insulin resistance or neurodegenerative disease has not been assessed. Altogether, these data add astrocyte insulin signaling to the ever-growing list of ways that astrocytes and neurons interact and impact each other's function.

A1.5. BRAIN INSULIN RESISTANCE AND METABOLISM IN DISEASE

A1.5.1. Molecular contributors to brain insulin resistance

A number of mechanisms have been proposed as potential factors in the development of brain insulin resistance. When we consider the complexity and heterogeneity of diseases associated with insulin resistance, it becomes clear that there is undoubtedly an interplay between multiple mechanisms *in vivo*. For example, eating a diet that has a high fat content, living a sedentary lifestyle, and genetic predisposition are all risk factors for the development of insulin resistance and type 2 diabetes (T2D) in humans. Although many of these are also risk factors for the development of cognitive decline and AD, the brain insulin resistance of AD is also present in individuals without diabetes[39].

Below we will explore some of the mechanisms thought to contribute to brain insulin resistance, mostly through the lens of high-fat diet in mice. Mouse studies of HFD-induced obesity and insulin resistance have found that the composition of the HFD, including the source of fat, has a significant impact on the degree of insulin resistance. HFD can result in increases in free fatty acids (FFAs), ceramides, phosphatidic acid and diacylglycerols, all of which have been implicated in peripheral insulin resistance[489]. Of course, humans rarely, if ever, eat a diet that is as consistent and molecularly defined as

what mice are given in HFD studies. Despite these considerations, there is much to be learned from mouse studies about how insulin resistance develops, both throughout the body and in the brain, and how this contributes to metabolic defects and neurodegeneration. Additionally, while not discussed in more detail below, it is important to note that there is likely at least some contribution of impaired insulin transport into the brain in response to HFD and during neurodegenerative diseases associated with brain insulin resistance. The extent to which disrupted insulin transport into the brain or any of the individual molecular mechanisms described below contribute to cognitive impairment and insulin resistance in the brain compared to any of the other mechanisms is unknown. It is critical to keep all of these considerations in mind when discussing and studying brain insulin resistance in any given neurological context.

A1.5.2. Fatty acids and inflammation

One of the primary mechanisms proposed for the development of brain insulin resistance is the accumulation of damaging FFAs and ceramides in the brain as a result of HFD intake. Indeed, chronic brain infusion of saturated fatty acids, such as palmitic acid (PA), leads to insulin resistance[490]. Elevated levels of circulating FFAs induce the synthesis of ceramides in the brain[45,491,492]. Excessive brain ceramides induce inflammation through activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, which coordinates transcription of proinflammatory cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor α (TNFα), and IL-6. These inflammatory mediators not only impair insulin signaling on their own, but also activate c-Jun N-terminal kinase (JNK), a signaling molecule involved in the endoplasmic reticulum (ER) stress response that disrupts brain insulin signaling (Figure A1.3)[45,493,494]. Importantly, Schell and colleagues[495] demonstrated that varied compositions of HFDs

have differential effects on the induction of insulin resistance, JNK activation, and mitochondrial function in the hypothalamus. This research highlights the importance of matching the macronutrient composition of control and experimental diets and testing different types of HFDs (Western vs. Mediterranean, etc.) to better elucidate which components of the HFD may be influencing the onset of insulin resistance.

Palmitic acid, which is the most abundant saturated fatty acid in most HFDs used for rodent research, is increased in the cerebrospinal fluid and brains of individuals with obesity[492,496,497], as well as the hypothalamus of mice fed a long-term HFD[494]. Treating neurons *in vitro* with PA leads to insulin signaling deficits, inflammation, and JNK activation in these cells[494,495]. Primary astrocytes treated with PA have similarly elevated astrogliosis and inflammatory cytokine expression[494]. Interestingly, inducible KO of a key inducer of NF-κB, IKKβ, from astrocytes after the onset of HFD-induced obesity and astrocyte reactivity reduces food intake and prevents further weight gain in these mice[498]. Furthermore, mice with IKKβ KO in astrocytes have elevated energy expenditure, glucose tolerance, and insulin sensitivity, all of which coincide with diminished hypothalamic inflammation[498]. In support of this, overexpression of IKKβ and NF-κB led to weight gain coupled with glucose intolerance and insulin resistance[499]. These studies suggest that insulin resistance, induced by either a HFD or elevated concentrations of PA, is detrimental to astrocytes due to induction of an inflammatory state. Indeed, astrogliosis has been observed in obese humans as well[406,500–502]. Whether the inflammatory response of astrocytes to a HFD plays a direct role in influencing whole-body energy expenditure, food intake, glucose tolerance, and insulin sensitivity or if it instead acts indirectly by influencing neuronal populations that are

involved in controlling these processes is as of yet unclear.

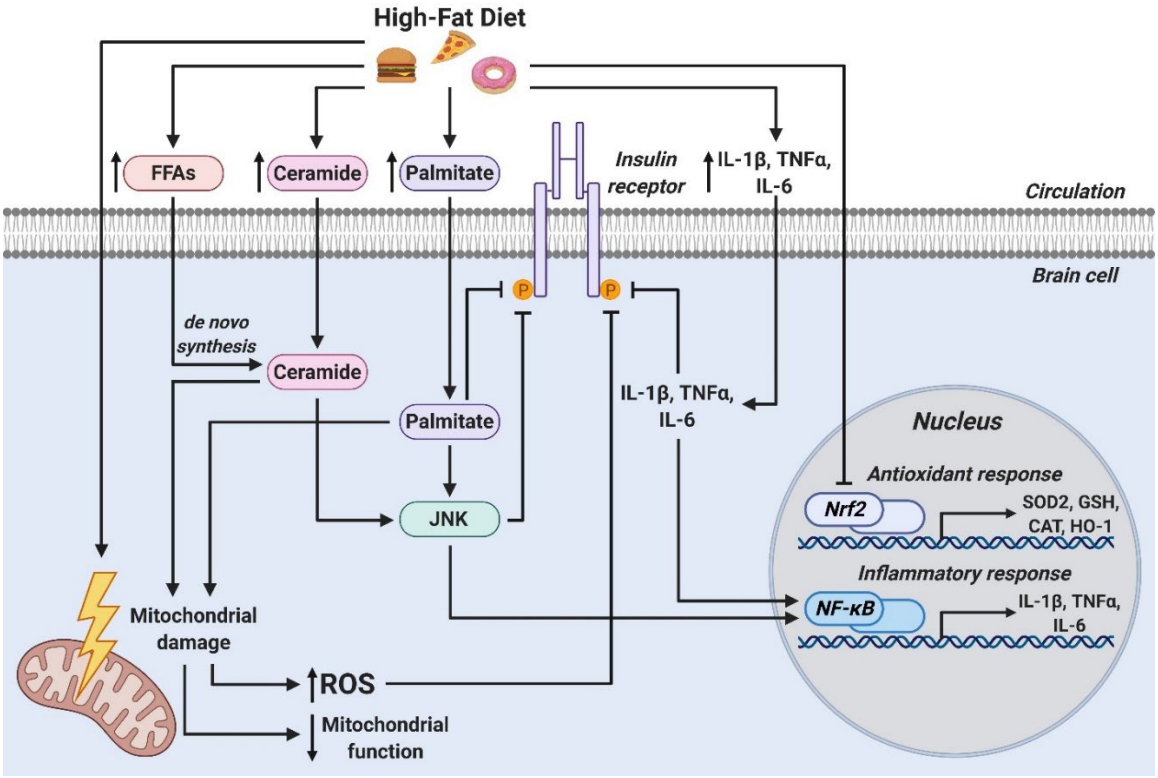


Figure A1.3. Mechanisms of brain insulin resistance. High-fat diet feeding leads to elevated circulating levels of FFAs, ceramides, palmitate, and inflammatory cytokines. All of these are transported into the brain, where they impair insulin receptor signaling, activate ER stress signaling through JNK, and initiate an inflammatory response via NF-κB signaling. HFD feeding, palmitate, and ceramide all induce mitochondrial damage, resulting in elevated ROS production and diminished mitochondrial function. The increase in ROS production coupled with HFD-induced impairment of the Nrf2-driven antioxidant response culminates in oxidative stress, which further exacerbates insulin resistance. CAT-catalase, GSH-glutathione, FFAs-free fatty acids, HFD-high-fat diet, HO-1-heme oxygenase-1, IL-1β-interleukin-1β, IL-6-interleukin-6, JNK-c-Jun N-terminal kinase, NF-κB-nuclear factor kappa-light-chain-enhancer of activated B cells, Nrf2-nuclear factor erythroid 2-related factor 2, ROS-reactive oxygen species, SOD2-superoxide dismutase 2, TNFα-tumor necrosis factor α.

A1.5.3. Oxidative stress

As a natural byproduct of mitochondrial oxidative metabolism and some enzymatic reactions, ROS have been implicated in the control of several cellular functions, including cell death, cell signaling, induction of antioxidant responses, and regulation of mitochondrial metabolism. Indeed, mitochondrial ROS are critical for many major signaling pathways, including NF-κB, JNK, and insulin among others[503–507]. Excess free radicals are normally neutralized by a variety of antioxidants and enzymes that together form the antioxidant response. While more nuanced and extensive than will be discussed in this review, the antioxidant response is largely controlled by the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), which regulates the expression and activity of many key antioxidants and antioxidant biosynthesis pathways[508] including superoxide dismutases (SODs), glutathione (GSH), HO-1, and catalase (CAT). The short polypeptide antioxidant GSH is used as a cofactor for antioxidant enzymes, such as glutathione peroxidases, that are critical for reducing ROS and lipid peroxides[509]. In addition, many of these are localized within mitochondria in order to regulate mitochondrially-derived ROS. All of these antioxidants maintain cellular redox balance and prevent oxidative damage to essential components of the cell. Oxidative stress occurs when there is an imbalance in the ROS-antioxidant axis, resulting in damage to the cell which eventually can culminate in cell death.

Oxidative stress is prevalent in an array of diseases, including obesity, T2D, and AD. Much of the research into the connections between oxidative stress and insulin resistance have utilized skeletal muscle as a model tissue, however the role of ROS in the development of brain insulin resistance has more recently gained attention. The brain is particularly sensitive to oxidative damage due to its high oxygen utilization and

relatively low antioxidant activity relative to other tissues[45–47]. At the same time, the presence of insulin resistance in the brain has been linked to increased oxidative stress and altered antioxidant expression and activity, as well as elevated levels of protein, lipid, and DNA oxidation products (Figure A1.3)[45,510–513]. Treating cultured neurons with PA as a means of mimicking aspects of HFD feeding induces both oxidative stress and NF-κB signaling[45,495,514,515]. Ceramide exposure in neurons also leads to oxidative stress, insulin resistance, and mitochondrial dysfunction[45,516,517]. Further, disrupting mitochondrial homeostasis in the hypothalamus through knockdown of the mitochondrial chaperone Hsp60 is sufficient to induce insulin resistance, mitochondrial dysfunction, and ROS production[518].

Feeding mice a HFD, which increases whole-body insulin resistance (as measured by homeostatic model of insulin resistance; HOMA-IR) positively correlates with brain ROS production, while negatively correlating with brain mitochondrial ATP production[486,512]. Additionally, Ruegsegger et al.[486] found decreased activity of the antioxidant enzymes SOD2 and CAT in whole-brain lysates of mice fed a HFD for 4 weeks, consistent with some previous reports[519], but in disagreement with others[512]. The latter study, however, placed mice on a HFD for 8 weeks and also showed regional differences in antioxidant enzyme expression and activity[512]. Together, these findings demonstrate the importance of taking into account the length of the HFD and the brain regions assessed when making conclusions regarding oxidative stress and HFD-induced brain insulin resistance. Nonetheless, the positive correlation between HOMA-IR in mice and ROS production in the brain is notable. Altogether, it is clear that oxidative stress is an important aspect of brain insulin resistance that can influence and be influenced by

the lipid content, and inflammatory and metabolic states of the brain during insulin-resistant conditions.

A1.5.4. Alzheimer's disease and insulin resistance

Insulin resistance in the brain has been observed in association with HFD feeding, obesity and T2D[486,520,521]. Interestingly, it has also been associated with several neurologic disorders, including depression, Parkinson's disease, cognitive decline, and AD[522]. In AD, brain insulin resistance is a prominent characteristic and potential factor in the onset, independent of coincident T2D[39,523]. Studies looking at brains from recently deceased AD patients and age-matched controls found that IR and IGF1R expression are significantly reduced in multiple regions of the AD brain[524] and components of the insulin signaling cascade are decreased in both T2D and AD[525]. Moreover, *ex vivo* stimulation of AD brains with insulin or IGF1 revealed that signaling is impaired for both hormones in AD[39]. However, both T2D and excess caloric intake are also risk factors for developing cognitive deficits and AD[526–528] and the exact molecular mechanisms linking insulin resistance to AD are not currently well-characterized. It remains unclear if the insulin resistance seen in the brain in AD occurs by the same mechanisms as T2D and HFD feeding. Wakabayashi et al.[529] tried to address this question by comparing HFD feeding to IRS2 KO in an AD mouse model that induces amyloid-β (Aβ) accumulation. They found that AD mice fed a HFD were insulin resistant prior to the onset of amyloid pathology and that HFD feeding exacerbated the rate of Aβ accumulation[529], consistent with previous studies[530,531]. They also found that KO of IRS2, while inducing insulin resistance in the brain, liver, and pancreas, diminished Aβ deposition in the brain, suggesting that HFD feeding and IRS2 KO act through different mechanisms to impact AD pathology[529].

Although the underlying pathogenesis of the disease is still unclear, the core pathological features of AD are extracellular plaques composed of Aβ and accumulation of phosphorylated tau into intracellular tangles. Insulin signaling appears to be important for mitigating amyloid plaques in animal and cellular models of AD and also regulates normal clearance of Aβ oligomers. Whole-brain IRKO mice (NIRKO) have increased levels of phosphorylated tau, which is a critical component for the generation and accumulation of neurofibrillary tau tangles[476]. Supplementation with either insulin or IGF1 decreases cognitive deficits in an AD mouse model and increases trafficking and clearance of Aβ[532–535]. Furthermore, insulin is able to ameliorate the Aβ oligomer-induced impairment to long-term potentiation in hippocampal brain slices[536]. Studies conducted on primary neurons have found that treating the cells with Aβ oligomers induces local insulin resistance by reducing the amount of IR on dendrites prior to dendritic spine loss, and sequestering the IRs to the neuronal soma[537,538]. Treating these cells with insulin stopped the loss of synapses induced by Aβ oligomer exposure, although it should be noted that the concentration of insulin used in this study is high enough to activate both IR and IGF1R, possibly suggesting that IGF1R signaling is involved in preventing neuronal synapse loss in response to Aβ oligomers[386,537,539].

How and when insulin resistance arises during the course of cognitive decline and AD pathology is not currently well known, however Aβ, ceramides, ROS, and inflammation have all been suggested as mechanisms that can lead to furthering the progression of AD[513,531,532,540–542]. It is likely that the development of insulin resistance in AD is multifactorial and highly complex.

A1.5.5. Impact of brain insulin resistance on brain metabolism

As diseases associated with insulin resistance are primarily metabolic disorders, it is not surprising that metabolism and mitochondrial function in the brain are impaired in these conditions. HFD-induced brain insulin resistance leads to decreased OXPHOS and TCA cycle function in the hypothalamus, hippocampus, and cortex of mice, which coincides with reduced mitochondrial content and mRNA expression of OXPHOS components[486]. In addition, insulin resistant rodents have increased ROS production and impaired mitochondrial oxygen consumption in the brain, resulting in reduced ATP production and mitochondrial dyshomeostasis[45,543–545]. These effects are also observed in models of AD, where excess ROS and disruptions to both mitochondrial function and quality control are prominent aspects of the disease[546,547].

Another contributing factor to the TCA cycle and overall mitochondrial bioenergetic function is the oxidation of fatty acids. Fatty acid β-oxidation is a metabolic pathway that utilizes fatty acids to generate acetyl-CoA, which is then fed into the TCA cycle. In the brain, this process is necessary to minimize the damaging accumulation of lipids. Evidence for this comes from models aimed at disrupting hypothalamic fatty acid sensing and oxidation. Knockout of carnitine palmitoyltransferase 1c (CPT1c), a brain-specific isoform of the enzyme necessary for transport of long-chain fatty acyl-CoAs into the mitochondria for FAO, decreases food intake and body weight[548]. This suggests that CPT1c activity in the brain modulates feeding behaviors and possibly body fat accumulation. In support of this, increasing the amount of malonyl-CoA, a fatty acid synthesis intermediate and inhibitor of CPT1c and FAO, also reduces food intake[549]. Interestingly, astrocytes appear to utilize FAO as an energy source much more than neurons[435], which may in part be due to their position at the BBB and resulting exposure

to circulating fatty acids. When FAO is overwhelmed by chronic HFD intake, increased PA in the brain impairs astrocyte lipid sensing and uptake[550]. This deficiency in astrocyte lipid uptake can lead to hypothalamic insulin resistance and accumulation of ceramides[492,550,551]. While the pathways are still not fully elucidated, cellular and mitochondrial metabolism in the brain are clearly impacted by insulin resistance.

In regards to mitochondrial quality control, some studies show that mitochondrial fission in the hippocampus is increased in response to HFD feeding[486]. Indeed, using an inhibitor of Drp1 to decrease mitochondrial fission prevents HFD-induced insulin resistance in the dorsal vagal complex of mice[519], a brain region involved in regulation of hepatic glucose production[552]. Furthermore, inhibition of Drp1 activity in primary hippocampal neurons isolated from obese (*ob/ob*) mice ameliorated the obesity-related decrease in ATP production[553], suggesting that Drp1-mediated fission may underlie deficits in neuronal ATP production in this model. Pharmacological inhibition of Drp1 in these obese (*ob/ob*) mice restored hippocampal synaptic plasticity, linking excess mitochondrial fission to obesity-related cognitive deficits[553]. However, there is likely a heterogeneous mitochondrial response to HFD feeding, as AgRP neurons have elevated mitochondrial fusion after long-term HFD exposure, which coincides with a higher AgRP firing rate[40,554,555](Figure A1.2). When mitochondrial fusion is impaired in these neurons by AgRP-specific MFN1 or MFN2 KO, the mice are protected from the effects of HFD feeding. These mice demonstrate increased mitochondrial fission in AgRP neurons, leading to diminished ATP production, action potential firing, food intake, and fat mass, while increasing whole-body insulin sensitivity[40]. Meanwhile, disrupting mitochondrial fusion in POMC neurons impairs their ability to regulate whole-body metabolism[41,43]. Putting all of these data together, it is clear that there are likely cell-type-specific

responses to insulin resistance and nutrient availability, as well as region-specific responses. As such, studies assessing overall levels of mitochondrial fission or fusion in whole brain regions during insulin resistance may be missing important information regarding the cell-type specificity of these responses. Furthermore, how changes in mitochondrial dynamics due to HFD feeding and insulin resistance differ across brain regions requires additional research.

Altogether, the metabolic impact of insulin resistance and impaired insulin signaling in the brain is extensive. Abnormalities to mitochondrial bioenergetics and dynamics have been observed in models of both insulin resistance and AD *in vitro* and *in vivo*. The contribution to these defects and whether metabolism in distinct cell types is differentially affected by insulin resistance and insulin-resistant diseases requires further exploration. Furthermore, identification of how insulin modulates metabolic processes in the brain and in individual cell types, and how these are affected by insulin resistance may yield novel targets for therapeutic intervention.

A1.6. THE THERAPEUTIC POTENTIAL OF DIABETES TREATMENTS FOR NEUROLOGIC DISORDERS

A1.6.1. Insulin

With the knowledge that the brain is insulin resistant in Alzheimer's disease, and that improving insulin signaling in the brain in AD mouse models ameliorates the disease, a variety of approaches have been utilized to try and overcome the insulin signaling defects of AD. These interventions lean heavily on our knowledge of treatments for type 2 diabetes. Trials to date can be thought of broadly as attempts to

either overcome insulin resistance with additional insulin, improve insulin sensitivity, or a combination of the two.

While it is known that impaired insulin signaling occurs in the brain parenchyma as a result of excess FFAs, inflammation, and ROS production, insulin transport across the BBB may also be diminished during insulin resistance. Both IR downregulation and altered IR signaling by brain endothelial cells could reduce insulin uptake into the brain; however, studies have shown that BBB integrity during insulin-resistant conditions is actually decreased, allowing for the passage of more solutes into the brain[556]. Human data examining CSF insulin levels in AD have been mixed. An early study found that CSF insulin was reduced in moderate to severe AD, despite increased blood insulin levels[557], but a more recent study found no correlation between disease state and CSF insulin[558]. Further, in that second study, a higher CSF insulin concentration was associated with worse cognition and increased phosphorylated Tau in women. This stands in contrast to a series of studies in which inducing hyperinsulinemia with hyperinsulinemic-euglycemic clamps actually improved cognition in patients with AD[559]. The clamp studies however, rather than being an observed correlation, stood as a potential intervention.

In T2D management, high doses of systemic insulin are often required to overcome insulin resistance and normalize blood glucose levels. This same approach cannot be used in AD patients without diabetes to overcome brain insulin resistance, as systemic treatment with insulin would result in hypoglycemia. To circumvent this issue, AD intervention trials were designed that delivered insulin directly to the brain through intranasal administration. Initial studies showed that delivery of intranasal insulin had the potential to improve both cognition and AD biomarkers[560–563]. These data were in line

with studies which also demonstrated enhancements in memory in cognitively normal individuals receiving intranasal insulin[564]. Unfortunately, a recent phase 2/3 clinical trial designed to prove efficacy of intranasal insulin for the treatment of AD failed to show a meaningful difference between treatment groups[565]. The authors cited issues with the delivery device as a concern in the study, but there are a variety of other factors that could have contributed to the outcome. There may be significant differences in the degree of brain insulin resistance in a given individual, as is observed in the periphery. If that is the case, then individualized dosing could be required, but we currently have no way to determine how insulin resistant a given person's brain might be. While this might lead one to conclude that higher doses would be better, increased insulin binding to the IR leads to receptor downregulation, thus potentially worsening the resistance[566]. Further complicating dosing of insulin in the brain, there is actually a greater abundance of IGF1R in the brain compared to IR. Both insulin and IGF1 can bind to each other's receptors and, like the IR, the IGF1R is also downregulated in AD[39]. Increased IGF1R signaling has also been shown to improve AD in mouse models[533]. If IGF1R is playing an import part in AD pathology, then even higher doses of insulin would be required due to the lower affinity of insulin for the IGF1R. Paradoxically, decreasing IGF1R signaling has also been beneficial in mouse models of AD, demonstrating a need for further research and understanding of the role IGF1R signaling plays in AD pathology[567].

A1.6.2. Insulin sensitizers

Another approach that has been attempted in recent years is to improve brain IR sensitivity. Large studies have been conducted with both the biguanide metformin and the thiazolidinedione (TZD) pioglitazone. Epidemiological data relating metformin to the prevention of AD have been mixed, though generally favoring a protective effect[568–571].

The mechanism of action of this drug is not entirely understood, however it seems to improve insulin sensitivity at the liver and improve AMP-activated protein kinase (AMPK) signaling[572]. It also has the often over-looked side-effect of causing vitamin B12 deficiency in some patients[573]. Given the relationship between vitamin B12 deficiency and cognitive impairment, this may be a reason for some of the confounding results with metformin in the prevention of AD. Several prospective intervention trials are being planned or are underway to systematically look at the potential of metformin to impact aspects of aging, including cognitive dysfunction.

Pioglitazone, in contrast to metformin, has a well-defined mechanism of action. It is a peroxisome proliferator-activated receptor γ (PPARγ) receptor agonist. Activation of the receptor leads to lipogenesis, removing FFAs from circulation and thus improving insulin sensitivity. This medication is a very potent insulin sensitizer and does not cause hypoglycemia in people without diabetes, making it an attractive candidate for the prevention of AD. However, a large prospective clinical trial to evaluate pioglitazone for the prevention of mild cognitive impairment was terminated early for lack of efficacy (NCT01931566). It is perhaps not all that surprising that pioglitazone was unsuccessful in preventing cognitive decline in a non-diabetic patient population. While brain insulin resistance in AD has been observed, it seems likely that Aβ and local inflammation are also important drivers of brain insulin resistance that are unlikely to be impacted by pioglitazone. While pioglitazone may be able to reduce the added burden of AD seen in people with obesity and diabetes, who are more likely to have a contribution to insulin resistance from dyslipidemia, this has not been directly tested.

More recently, there has been interest in using a newer class of diabetes drugs, the sodium glucose transporter 2 (SGLT2) inhibitors, for the prevention and/or treatment

of Alzheimer's disease. This drug class reduces blood glucose by inducing glucosuria, thus their mechanism of action is independent of insulin. The impact of glucose lowering, however, may serve to reduce brain insulin resistance[574,575]. Epidemiologic evidence supports a potential benefit from this drug class on cognitive decline in patients with diabetes[576], but prospective clinical trials have not been performed. Interestingly, these drugs also cause an increase in circulating levels of ketone bodies, a preferred energy source for the brain. There are currently two small clinical trials underway to test the impact of this drug class on brain function, one with a focus on ketone body production in normal subjects (NCT03852901) and the other testing cognitive function in patients diagnosed with Alzheimer's disease (NCT03801642).

A1.6.3. Combination therapy

The two most successful interventions to slow AD to date which address brain insulin resistance might be considered combination therapies in which insulin sensitivity and insulin secretion are likely both improved through the intervention. These include lifestyle interventions and the glucagon-like peptide-1 (GLP1) receptor agonist dulaglutide. The landmark FINGER study (Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability) demonstrated that targeting diet and exercise, but also cognitive and social activities and vascular risk factors, could significantly prevent cognitive decline in a high-risk population[577]. The FINGER study was not designed to isolate the effects of any of the individual interventions. While brain insulin signaling may well have improved using this multi-factorial approach, other risk factors were also mitigated, likely contributing to the positive result.

Finally, the GLP1 receptor agonists are examples of drugs that improve insulin secretion by binding to the GLP1 receptor on pancreatic beta cells and stimulating

insulin release, while also decreasing insulin resistance, albeit indirectly, by binding GLP1 receptors in the hypothalamus to induce weight loss. In addition to the potential beneficial impact of these drugs on insulin signaling, GLP1 receptors are expressed throughout the brain and are able to directly impact cellular metabolism[578]. Dulaglutide, one drug in the GLP1 receptor agonist class, was found in a clinical trial to reduce the risk of developing AD[579]. While this drug and/or drug class may positively impact brain metabolism and the risk for AD, there are many caveats worth noting. First, this was an exploratory analysis of a trial designed to look at cardiovascular outcomes, not cognitive outcomes. In addition, in contrast to the many studies mentioned above, this trial only enrolled patients with diabetes. Patients in the treatment group had better diabetes control, lost weight and had lower blood pressure and fewer adverse cardiovascular events throughout the 5 years of follow up[580]. Thus, once again it is difficult to separate out potential impacts on brain metabolism from improvements in vascular risk. As we consider the successes and failures in addressing the contribution of brain insulin resistance to the pathogenesis and treatment of AD, it seems clear that there is still a great deal of research needed to understand the underlying causes of abnormal brain insulin receptor signaling in this disorder.

A1.7. CONCLUSION

In the 100 years since the introduction of insulin for clinical care, our knowledge of how this hormone works has grown immensely. Despite all of this progress, our knowledge of the action of insulin in the brain still lags well behind the rest of the body. As we continue to further delineate the role of insulin signaling in the brain, it is our hope that this will eventually lead us towards new therapies for obesity, diabetes, and neurodegenerative diseases.

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