The cholesteryl transfer protein and apolipoprotein E in Alzheimer's disease models

Sasen Efrem

Department of Pharmacology and Therapeutics
McGill University, Montréal, Quebec
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To my parents

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Abstract

Alzheimer's disease (AD) is the most common form of dementia affecting about 500,000 people in Canada and 24 million people worldwide. As AD has been known to be heavily related to lipid & cholesterol metabolism both in the brain and in the periphery, as evidenced by both high plasma LDL levels and increased occurrence of the apolipoprotein E ε4 polymorphism being well-established risk factors, it is important to develop models highly aligned to the human AD profile, particularly in terms of lipid metabolism-related processes. Our lab found that when cholesteryl ester transfer protein (CETP), a protein that shuttles cholesteryl esters from HDL particles to VLDL and LDL particles, is expressed in mice under the human promoter, a humanized profile of rodent lipoprotein particles occurs. Remarkably, when CETP mice were crossed with the Thy1-APP Tg Alzheimer mouse model expressing human APP, a 5-fold increase in soluble and insoluble Aβ levels in the brains of double transgenic mutants were seen. In my MSc research, I found that human apolipoprotein E4 (hApoE4)-expressing astrocytes have larger and more lipid droplets compared to human apolipoprotein E3 (hApoE3)-expressing astrocytes. Furthermore, I found that recombinant CETP (rCETP) can modulate astrocytic lipoproteins in cell-free systems in dependence of hApoE isoform and CETP activity. Despite this, neither secreted CETP from stably CETP-expressing hApoE astrocytes nor exogenous rCETP had activity-dependent effects on Aβ levels in astrocyte-SY5Y co-culture systems. Finally, in my master's thesis, I developed and characterized liver-specific CETP viruses with high *in vivo* infectivity, which can be used for future analyses of whether peripheral CETP confers changes in brain Aβ production and cholesterol/lipoprotein profiles in both the CNS and periphery.

Abrégé

La maladie d'Alzheimer (MA) est la forme la plus courante de démence, touchant environ 500 000 personnes au Canada et 24 millions de personnes à travers le monde. Comme il est connu que la MA est étroitement liée aux métabolismes des lipides et du cholestérol dans le cerveau et sa périphérie, (c.-à-d. des forts taux de lipoprotéines de basse densité (LDL) et des taux élevés de polymorphisme de l'ApoE4 sont tous deux des facteurs de risque bien établis) il est important de développer des modèles hautement alignés au profil humain de la MA, notamment en ce qui concerne les mécanismes du métabolisme lipidique. Notre laboratoire a conclu que lorsque la protéine de transfert des esters de cholestérol (CETP), une protéine qui stimule le transfert des esters de cholestérol des particules HDL aux particules VLDL et LDL, est exprimée dans les souris sous un promoteur humain, un profil humanisé des particules de lipoprotéine des rongeurs en survient. Étonnamment, lorsque les souris CETP furent croisés avec le modèle murin de l'Alzheimer 5xFAD exprimant de l'APP humain, une augmentation de 10 fois des niveaux des Aβ solubles (largement admis comme étant un marqueur pathognomonique primaire de la maladie) ainsi qu'une augmentation de 5 fois des niveaux de Aβ insolubles dans le cerveau de double mutants transgéniques ont été constatés. Dans le cadre de mon projet de maîtrise, j'ai trouvé que les astrocytes exprimant hApoE4 ont des gouttelettes lipidiques plus grosses et plus nombreuses que les astrocytes exprimant hApoE3. De plus, j'ai observé que la protéine de transfert des esters de cholestérol recombinante (rCETP) peut moduler les lipoprotéines astrocytaires dans des systèmes acellulaires selon l'activité de l'isoforme hApoE et de la CETP. Malgré cela, ni la CETP sécrétée par les astrocytes hApoE exprimant la CETP de façon stable, ni la rCETP exogène n'avaient d'effets dépendant de l'activité sur les taux de Aβ

dans les systèmes de co-culture astrocyte-APP-SY5Y. Enfin, dans ma thèse de maîtrise, j'ai développé et caractérisé des virus CETP spécifiques du foie avec un pouvoir infectieux in vivo élevé, qui pourront être utilisés pour de futures analyses qui permettront de déterminer si la CETP périphérique confère des modifications de la production d'Aβ cérébrale et de caractériser les profils de cholestérol/lipoprotéines dans le système nerveux central et périphérique.

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Contributing authors

The experiments conducted in this thesis were performed by myself with supervision by Lisa Munter. Preliminary *in vitro* and *in vivo* data were conducted by Felix Oestereich. Biotinylation of the Anogen Aβ detection antibody for sandwich-based ELISA was also prepared by Felix Oestereich. hApoE astrocytes were provided as a gift by David Holtzman, APP-expressing SH-SY5Y cells were developed by Lisa Munter. CETP plasmid Sanger Sequencing and astrocyte genotyping was performed by the McGill University and Génome Québec Innovation Centre. The AAVDJ-hTGBprom-CETP(WT)-T2A-EGFP-WPRE virus was developed by Neurophotonics Centre in Quebec, whereas the AAV-TGB-CETP virus plasmid was developed by Daniel J. Rader. Size-exclusion chromatography assistance and training was provided by Michal Zelinski.

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Introduction

Alzheimer's disease

History

On November 3rd, 1906, Dr. Alois Alzheimer, a German psychiatrist and neuropathologist, first reported on "a peculiar severe disease process of the cerebral cortex" (Über einen eigenartigen, schweren Erkrankungsprozeß der Hirnrinde) to the 37th meeting of South-west German Psychiatrists in Tubingen November, 1906 (Hippius, 1998). He described the long-term study of his patient Auguste Deter, the first documented case of this disease who initially presented with symptoms of dementia such as loss of memory, confusion, delusions, and difficulty with speech. This disease, which would later bear Dr. Alois Alzheimer's name, progressed aggressively and Deter remained at the Municipal Asylum for Lunatics and Epileptics in Frankfurt until her death in April, 1906. Post-mortem, Dr. Alzheimer found that Deter's brain had extensive atrophy along her cerebral cortex, lipid deposits, neurofibrillary tangles as well as senile plaques which he described as "deposition of a special substance"—characteristics that he linked to dementia (Hippius, 1998; Zhan et al., 2015). Now, some of these characteristics have become hallmarks of Alzheimer's Disease (AD), and more than a century since, research to understand and cure the disease have accelerated to become a global effort.

Alzheimer's Disease: An Overview

Alzheimer's disease is the most common form of dementia and the seventh leading cause of death in Canada (Statistics Canada, n.d.). Genetically, the disease is divided into familial and sporadic cases, the former being due to mutations in amyloid precursor protein (APP), presenilin 1 (*PSN1*) and presenilin 2 (*PSEN2*) while the latter is due to largely undefined genetic and

environmental factors (Piaceri, Nacmias, & Sorbi, 2013). While familial AD makes up only about 1-5% of AD cases, sporadic AD accounts for the remaining 95-99%; sporadic AD is thought to arise from complex interactions between susceptibility genes based on genome-wide association studies as opposed to dominant inheritance (Bali, Gheinani, Zurbriggen, & Rajendran, 2012). Worldwide, about 50 million people are living with dementia, and the total estimated global cost of dementia in 2018 is about US$1 trillion (Christina Patterson, 2018). These numbers are projected to at least triple by 2050 due to rising life expectancies and population ageing seen in most developed and developing countries (United Nations Development Programme, 2005). AD is already the third leading cause of death and disability for the elderly (individuals aged 65 and older), with cardiovascular diseases and malignant tumors being the first and second (Du, Wang, & Geng, 2018).

Mechanistically, AD is not well understood. What is known is that pathogenesis includes cholinergic neuron damage, oxidative stress and inflammation (Du et al., 2018). There exists somewhat of a dualism in the field of AD research with regard to proposed mechanisms of causality; most models of AD pathogenesis focus on either amyloid-beta (Aβ) accumulation or tau phosphorylation as being the key drivers or at least hallmarks for downstream manifestations of the disease (Christina Patterson, 2018; Du et al., 2018; Yun-wu Zhang, Thompson, Zhang, & Xu, 2011).

Since Alzheimer's discovery, in the past hundred years (as of 2018) only five drugs have been FDA approved to treat AD, all of which provide only temporary or incomplete symptomatic relief (Du et al., 2018). Thus, developing drugs that are more effective in treatment of AD is urgently needed for the near future.

The Amyloid hypothesis

The amyloid precursor protein (APP) is a single-pass transmembrane protein with largely unknown function expressed in many tissues of the human body (Uhlen et al., 2015). APP is highly expressed in the synapses of neurons where it is cleaved by several transmembrane proteases (Lanchec et al., 2017). The amyloid hypothesis looks at two cleavage pathways: the "non-amyloidogenic pathway" and the "amyloidogenic pathway".

APP can be cleaved through the non-amyloidogenic pathway wherein α-secretase cleaves APP within the Aβ region, releasing soluble APP-α (sAPP-α) and precluding Aβ generation (Yun-wu Zhang et al., 2011). The remaining membrane-bound fragment, APP-CTF83, can then be cleaved by γ-secretase resulting in the generation of P3 and the APP intracellular domain (AICD). Under the amyloid hypothesis, accumulation of Amyloid β (Aβ) in the brain is the primary driver of AD pathogenesis. Aβ is generated via the amyloidogenic pathway through sequential cleavage by first β-site APP-cleaving enzyme 1 (BACE1) followed by the γ-secretase complex (Figure 1.1). The γ-secretase complex is composed of four components, one of which is either of the above mentioned presenilins which constitute the catalytic subunit (De Strooper, 2007). The amyloidogenic pathway results in the generation of soluble APP-β (sAPP-β) post BACE1 cleavage, creating the APP-CTF99 membrane-bound fragment. This fragment is cleaved by γ-secretase releasing generating the AICD as well as an amyloidogenic Aβ fragment. These Aβ species are produced with varying lengths between 36-43 amino acids including two main alloforms having a length of 40 or 42 amino acids (Gregory & Halliday, 2005). Although Aβ40 is the main species secreted, Aβ42 is the more hydrophobic species associated with increased rates of aggregation and toxicity through nerve cell damage (M. P. Murphy & Iii, 2010; Yun-wu Zhang et al., 2011).

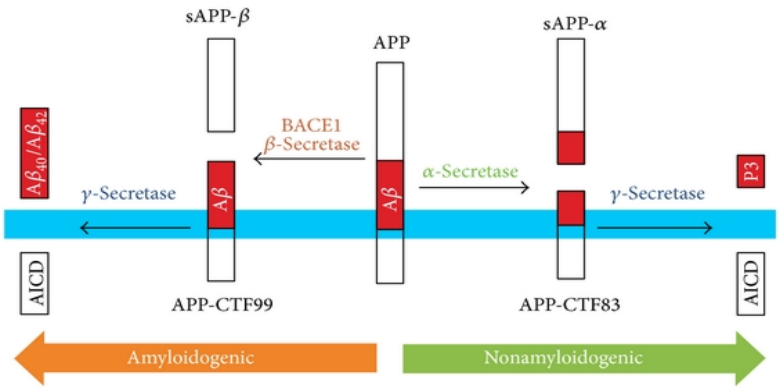


Figure 1.1: Amyloid precursor protein (APP) amyloidogenic and nonamyloidogenic processing pathways. Adapted from Wang et al. (Hui Wang, Megill, He, Kirkwood, & Lee, 2012)

The amyloid theory for AD pathogenesis is strengthened by the aforementioned familial AD-causing mutations in APP and presenilin, as well as a protective mutation in APP. Indeed, the Swedish mutation is a double point mutation in APP (APP K670M and APP N671L) first identified in two Swedish families who presented with AD that results in increased total Aβ42 levels (Mullan et al., 1992). Similarly, the London mutation (APP V717I), one of the most popular worldwide, is also associated with increased Aβ42 levels (Goate et al., 1991). Mutations in the presenilin 1 gene are the most common cause of familial AD, with over 150 mutations identified as of 2007 (De Strooper, 2007; Kelleher & Shen, 2017). Conversely, the Icelandic mutation in APP (APP A673T), found in Icelandic and Scandinavian populations, was found to be protective against age-related cognitive decline (Jonsson et al., 2012). This mutation results in both reductions in Aβ production and aggregation. Interestingly, the Swedish and Icelandic mutations are immediately flanking the BACE1 cleavage site of the amyloidogenic pathway implying that the mutations could directly influence rate of BACE1 cleavage (Kimura, Hata, & Suzuki, 2016).

Risk factors in Alzheimer's disease

The strongest risk factor for Alzheimer's disease is aging; most people with AD are 65 and older, and the risk of developing AD doubles every five years after this age (Jorm & Jolley, 1998). As well, genetics and family history constitutes a strong risk factor for Alzheimer's disease: this includes the mutations discussed above that are known to result in early onset autosomal dominant familial AD (EOAD), as well as other susceptibility genes for which polymorphisms have been reported to influence AD risk for late onset sporadic Alzheimer's Disease (LOAD). These genes include mutations in actin-like protein (*ACT*), LDL receptor-related protein (*LRP*) and, most notably, apolipoprotein E (*APOE*)—the strongest genetic risk factor for AD based on genome-wide association studies (Piaceri et al., 2013) (Motoshima, Goldstein, Igata, & Araki, 2006). In humans, there are three variants of the *APOE gene (APOE2, APOE3 & APOE4)*, with *APOE4* being associated with increased risk, *APOE3* being the most common and considered "neutral" in terms of AD risk, and *APOE2* being associated with decreased risk. Apart from genetic components, there are several lifestyle risk factors for LOAD including smoking, high blood pressure, diabetes, high cholesterol and lack of physical activity (Tariq & Barber, 2018).

Overview: cholesterol, triglycerides, phospholipids

Cholesterol

Cholesterol is an amphipathic molecule composed of four non-polar hydrocarbon rings and a polar hydroxyl group (at C-3) forming its steroid structure (Figure 1.2) (Goluszko & Nowicki, 2005). In its extended form, cholesterol is about as long as a 16-carbon fatty acid

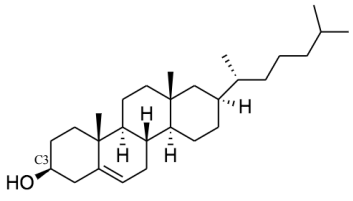


Figure 1.2: Structure of Cholesterol

(Tiwari, Rabia Alenezi, & Jun, 2016), making it a rather bulky lipid. Cholesterol and its derivatives are important for cell membranes structure and rigidity, as well as for the synthesis of bile acid, steroid hormones, vitamin D. At low temperatures, increased amounts of cholesterol in cellular membranes increase the cell membrane fluidity by intercalating between phospholipids and preventing rigid clustering. However, at high temperatures, increased amounts of cholesterol in cell membranes decreases cellular membrane fluidity as cholesterol is bulky in itself despite phospholipids being able to move more freely(Ohvo-Rekilä, Ramstedt, Leppimäki, & Slotte, 2002). Cholesterol is also often found in high concentrations within lipid rafts, which are lipid microdomains found on the plasma membrane (Korade & Kenworthy, 2008). These domains are enriched in sphingolipids and have functions in concentrating or separating membrane proteins within the bilayer, as well as regulating membrane trafficking and a plethora of cell signaling cascades (Korade & Kenworthy, 2008).

In humans, cholesterol is either produced *de novo* or supplied exogenously, predominantly from animal products in one's diet. The primary site of endogenous cholesterol production is the liver (Nemes, Åberg, Gylling, & Isoniemi, 2016). The synthetic pathway begins with 3 acetyl-CoA molecules condensing to form hydroxy β-methylglutaryl-Coenzyme A (HMG-CoA) upon HMG-CoA synthase activity. The next step, catalyzed HMG-CoA reductase (HMGCR), converts HMG-CoA to mevalonate. Following this, there are 8 additional reactions which convert mevalonate into squalene and finally to cholesterol. Cholesterol synthesis is primarily regulated four different ways, all of which are at the level of HMGCR. First, this enzyme is regulated by hormones including insulin and thyroxin which result in an upregulation of HMGCR while glucagon and glucocorticoids result in a downregulation. Secondly, HMGCR can sense high sterol levels and bind to insulin-induced gene 1 (INSIG) protein which in turn

will ubiquitnate the reductase with an Insig-bound ubiquitin ligase, gp78, and result in its proteasomal degradation (Dong, Tang, & Chen, 2012). As well, HMGCR reductase is upregulated by the sterol regulatory element binding protein (SREBP) through binding to the sterol regulatory element (SRE) on the *HMGCR* gene. During conditions of appropriate cellular cholesterol levels, SREBP, SREBP cleavage-activating protein (SCAP) and INSIG are bound to each other in complex in the ER (Figure 1.3). However, during periods of low cellular cholesterol, the SCAP and INSIG no longer bind to one another, and SCAP (still bound to SREBP) undergoes conformational changes that results in its packaging in COPII vesicles that move from the endoplasmic reticulum (ER) to the Golgi apparatus. In the Golgi apparatus, SREBP is cleaved by site-1 protease (S1P) followed by site-2 protease (S2P), which releases a cytoplasmic portion of SREBP that traffics to the nucleus and acts as a transcription factor to upregulate HMGCR, among other genes involved with increasing cellular cholesterol i.e. the Low-density lipoprotein receptor (Shao & Espenshade, 2014; X. Xu, So, Park, & Lee, 2013). Finally, HMGCR is regulated through adenosine monophosphate (AMP)-activated protein kinase (AMPK), which is thought to be a sensor for cellular energy. When the cell has low levels of adenosine triphosphate (ATP) and increased levels of AMP (such as during periods of low glucose, heat shock, and hypoxia) AMPK is activated and inactivates metabolic enzymes that are involved in ATP-consuming processes, including HMGCR (Motoshima et al., 2006).

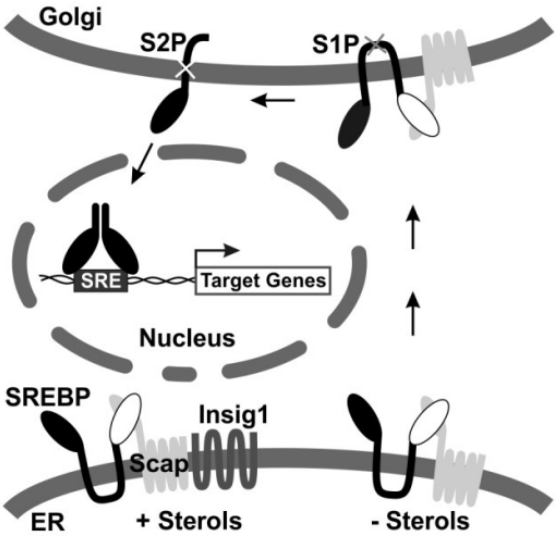


Figure 1.3: Schematic of SREBP maturation. Adapted from Yu et al. (Y. Yu, Pierciey, Maguire, & Alwine, 2013)

Triglycerides

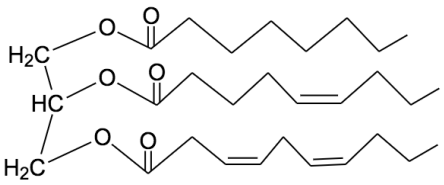


Figure 1.4: Representative structure of a triacylglycerol molecule.

A triglyceride molecule consists of a tri-esterified glycerol backbone and three fatty acid molecules (Figure 1.4). It represents the main constituent of dietary fat, vegetable fat, and fat deposits in animals (García-Palmieri & R., A. Cox, 1990). Triglycerides are commonly classified into saturated and unsaturated types, referring to the saturation of hydrogens in the fatty acids. While saturated triglycerides have no double bonds in any of the three fatty molecules, unsaturated triglycerides have one or many double bonds in these side chains, resulting in kinks within these hydrocarbon tails. As a result, saturated lipid molecules can pack more tightly to one another and these lipids are more likely to be solid at room temperature and have a higher melting point, while unsaturated lipids have a lower melting point (JM, JL, & L, 2002).

Triglycerides are derived either from the diet or produced *de novo* by the liver, predominantly (Y. Kawano & Cohen, 2013). Dietary triglycerides are first lipolyzed and emulsified into micelles in the small intestine, following which a hydrolyzation of ester bonds by pancreatic lipase yields free fatty acids and monoacylglyceride, which can be taken up by the small intestine. Once taken up by enterocytes of the small intestine, the triglyceride molecule is rebuilt. Some triglycerides are stored in lipid droplets, however most are packaged into chylomicrons by the microsomal triglyceride transfer protein (MTTP) and later secreted into the lymphatic system (Harchaoui, Visser, Kastelein, Stroes, & Dallinga-Thie, 2009). Other cell types including adipocytes and hepatocytes produce triglycerides *de novo* starting with glycerol being first phosphorylated by glycerol kinase, followed by acylation of two hydroxyl groups with fatty acyl-CoA molecules resulting in phosphatidic acid. From here, the remaining phosphate group is

removed by phosphatases and the final acylation step yields the triglyceride (Harchaoui et al., 2009).

Triglycerides play an important role as energy sources containing more than twice as much caloric energy as carbohydrates (Drummond & Brefere, 2014). When cells require increased amounts of blood glucose, hormones including glucagon and epinephrine are released which activate lipases to release free fatty acids from triglycerides stored in adipose tissue. These fatty acids enter the blood and are transported on albumin to cells which uptake fatty acids through fatty acid transport proteins such as SLC27 (Stahl, 2004). Once in the cell, a Coenzyme A (CoA) molecule is added to the fatty acid chain by long-chain-fatty-acid—CoA ligase resulting in an acyl-CoA molecule, which enters the mitochondria via the carnitine shuttle so that β-oxidation can occur in the mitochondrial matrix. The resultant acetyl-CoA produced by β-oxidation enters the citric acid cycle to yield ATP to use as energy (Houten & Wanders, 2010).

Phospholipids

Phospholipids consist of at least one fatty acid (generally two) esterified to a glycerol backbone at carbons one and two and a phosphate group with a hydrophilic residue such as choline or lecithin bound to carbon three (Küllenberg, Taylor, Schneider, & Massing, 2012). Phospholipids are therefore amphipathic consisting of a polar phosphate "head" group and two non-polar fatty acid tails (Figure 1.5). Phospholipids are the primary component of all cell membranes, which consists of a bilayer, or two "leaflets" of phospholipid sheets such that the interior and exterior sides of the bilayer is aqueous with the hydrophobic tails pointing into the middle of the sheets. Lipoprotein particles are surrounded by one layer of phospholipids such that fatty acid side-chains point into the middle of the particle, supporting a lipophilic

environment to store fats. Phosphatidylcholine accounts for more than 50% of the phospholipid in mammalian membranes (van Meer, Voelker, & Feigenson, 2008).

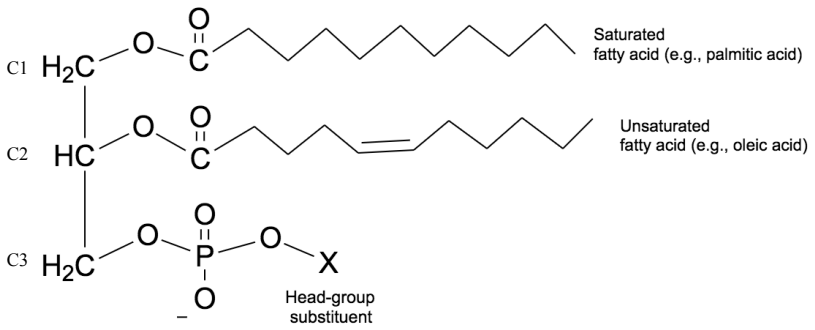


Figure 1.5: Representative structure of a phospholipid molecule.

It is widely believed that integral membrane proteins are arranged according to the fluid mosaic model, in which the phospholipid membrane acts as a two-dimensional fluid solvent and allows for free lateral diffusion and migration across the membrane (Singer & Nicolson, 1972). This fluidity can be impacted by cholesterol molecules, as previously mentioned.

Lipid metabolism and AD

Overview on Plasma lipoproteins

Cells require lipids like cholesterol, phospholipids and triglycerides for storing energy, cell membrane structural dynamics and cell signaling (Ohvo-Rekilä et al., 2002; van Meer et al., 2008). Lipoproteins are complex aggregates of lipids and proteins important for mobilization of these fats through the aqueous blood stream or extracellular space. Indeed, as lipids are not water-soluble, lipoprotein particles are vehicles to allow lipids to be transported from the hepatobiliary and gastrointestinal system to other tissues in the body. Lipoproteins are spherical or discoidal structures with a lipophilic interior containing neutral lipids (including triglycerides and cholesterol) and a hydrophilic outer shell composed of phospholipids and apolipoproteins (Apo). The apolipoproteins, density, size and lipid-to-protein ratio are used to classify

lipoproteins into distinct categories (Figure 1.6). Chylomicrons are the largest followed by very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), with high density lipoproteins (HDL) being the smallest (KR & C., 2018).

Lipoprotein metabolism is roughly divided into three pathways: the exogenous pathway, the endogenous pathway, and the reverse-cholesterol transport pathway (KR & C., 2018). The exogenous pathway starts with the absorption of dietary fats by the intestines, which gets packaged into the nascent triglyceride-rich chylomicron particles and secreted into the circulation. From here, HDL donates ApoE and ApoC-II and it is ApoC-II that activates lipoprotein lipase (LPL) which hydrolyzes core triglycerides into free fatty acids. These fatty acids in turn diffuse out of the hydrolyzed chylomicron into muscle or adipose cells. The chylomicron remnant continues to circulate in the bloodstream until it interacts via ApoE with chylomicron remnant receptors in the liver and is re-uptaken (García-Palmieri & R., A. Cox, 1990; KR & C., 2018). In the endogenous pathway, triacylglycerols and cholesteryl esters are assembled with ApoB-100 to form nascent VLDL particles and released into the bloodstream, particularly during periods of starvation. As seen with the chylomicrons, these nascent VLDL particles collect ApoE and ApoC-II from HDL to form the mature VLDL particle, and LPL hydrolyses triglycerides within the particles to release fatty acids that are then collected by peripheral tissues. These hydrolyzed VLDL particles are now IDL particles. From here, hepatic lipase (HL) will further hydrolyze IDL to LDL, which contain relatively high amounts of cholesterol. These LDL particles are absorbed by peripheral cells of target tissues or the liver. Endocytoses of LDL by these cells is done through ApoB-100 recognition through LDL receptors initiating clathrin-mediated uptake of these particles, eventually releasing cholesterol by lysosomal hydrolysis (García-Palmieri & R., A. Cox, 1990; KR & C., 2018; MacRae F et al.,

2000). Finally, there is the reverse cholesterol transport pathway, which results in the net movement of cholesterol from the periphery back to the liver through the smaller HDL particles. This pathway begins with the ApoA-I protein accepting cholesterol from peripheral tissues through the ATP-binding cassette transporter (ABCA1). The nascent HDL lipoprotein is discoidal in morphology. As excess cholesterol molecules are accepted, lecithin–cholesterol acyltransferase (LCAT) esterifies the cholesterol to cholesteryls and the HDL particle becomes more spherical as it matures. Once the HDL particle is mature and cholesterol-rich, it returns to the liver where the cholesterol is released and redistributed to other tissues or converted into bile. Unlike LDL, the mature HDL particle has ApoE as one of its minor apolipoproteins, along with VLDL, and chylomicrons. HDL particles are up-taken through the scavenger receptor class B type 1 (SR-B1) receptor in the liver (Marques et al., 2018).

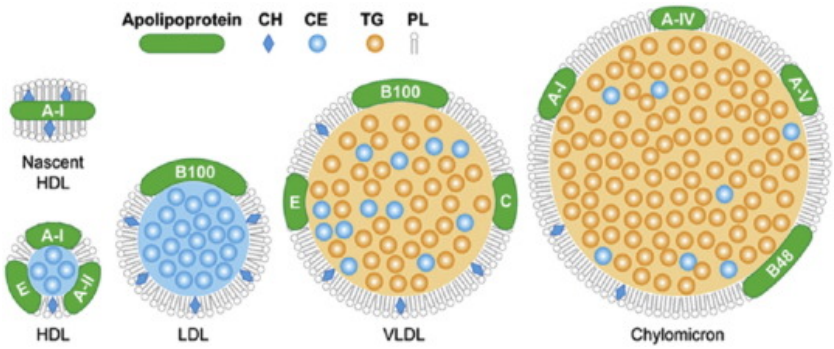


Figure 1.6: Schematic depiction of HDL, LDL and VLDL with major apolipoproteins. Reprinted (adapted) with permission from (Bricarello, Smilowitz, Zivkovic, German, & Parikh, 2011) Copyright (2011) American Chemical Society.

Lipoproteins, atherosclerosis and AD

Atherosclerosis is the underlying cause of several cardiovascular diseases including heart attacks and stroke. Many studies have identified cholesterol as a key component of arterial plaques, giving rise to the hypothesis that a disordered distribution of cholesterol between HDL

and LDL particles is central to atherosclerotic pathogenesis. Indeed, population studies have identified both increased LDL and ApoB-100 as directly associated with risk for atherosclerotic cardiovascular events (MacRae F et al., 2000). The proposed model starts with LDL being oxidized and taken up by macrophages. This process induces foam cell formation, which are fat-laden, unstable macrophages that accumulate on fatty deposits on arterial walls (Moore, Sheedy, & Fisher, 2013). ApoA-I, HDL or ApoE reduce atherosclerotic lesion formation by removing cholesterol from these macrophages and, in doing so, lowers oxidative stress and inflammation. As such, the reverse cholesterol transport system and higher HDL cholesterol levels are generally considered protective against development of atherosclerosis. In cases of high blood LDL cholesterol levels or unresolved inflammation, these foam cells develop into plaques which initiate inflammatory feedback cascades that lead to more foam cell recruitment and LDL modifications (MacRae F et al., 2000). These lesions may evolve with time and eventually become unstable, rupture and result in a thrombotic occlusion that underlies myocardial infarction and stroke (Moore et al., 2013).

Interestingly, since the early 1900s, atherosclerosis and poor vascular health have been implicated in AD pathogenesis as modifiable risk factors. Indeed, several studies have identified mechanisms by which cerebral vascular atherosclerosis can either increase Aβ production or decrease Aβ clearance from the brain (Gupta & Iadecola, 2015). Epidemiological evidence has indicated that hypertension, high LDL levels and low HDL cholesterol levels are being associated with increased amyloid deposition in the brain (Reed et al., 2014; Rodrigue et al., 2013). One study by Barnes and Yaffe has even suggested that at least half of AD cases worldwide are potentially attributable to vascular risk factors (Barnes & Yaffe, 2011). There are also countless clinical-pathological studies and *in vivo* experimental data using mouse models for

AD and atherosclerosis corroborating an association between poor cerebral vascular health and neuritic plaque burden or cognitive dysfunction (Gupta & Iadecola, 2015).

High plasma cholesterol and AD

In 1994, Sparks *et al*. were the first to report on a possible connection between cholesterol and AD (Sjögren & Blennow, 2005; Sparks et al., 1994). This group found that in the brains of rabbits fed a 2% cholesterol diet, there was moderate to severe accumulation of Aβ as compared to age- and sex-matched animals on a control diet that had virtually no Aβ plaque immunoreactivity (Sparks et al., 1994). This prompted several other studies looking at cholesterol involvement in AD including a series of experiments by Refolo *et al*. using APP-transgenic mice. In 2000, this group found that in mice with diet-induced hypercholesterolemia, using chow with 5% cholesterol, there were significantly increased levels of Aβ plaque deposits in the brain accompanied by significantly deceased levels of sAPP-α: the first APP cleavage product in the non-amyloidgenic pathway (Refolo et al., 2000; Yun-wu Zhang et al., 2011). In a follow-up study a year later, this group found that treating APP-transgenic mice with BM15.766, an inhibitor of 7-dehydrocholesterol-D7-reductase in the cholesterol biosynthetic pathway, there were reductions in Aβ in both the plasma and the brain by over two-fold (Refolo et al., 2001). There are also several *in vitro* studies corroborating this effect, wherein cholesterol depletion using statins strongly reduces or completely inhibits Aβ formation in cultured primary neurons (Fassbender et al., 2001; Simons et al., 1998).

Further, epidemiological data provide supporting evidence that high cholesterol levels associate with AD risk; several studies have found that high midlife total cholesterol levels (>6.5 mmol/l) were significant risk factors for AD, increasing risk as much as three-fold (Kivipelto & Solomon, 2006). Out of five long-term longitudinal studies on midlife serum cholesterol and

dementia (1. CAIDE Study, 2. Finnish Cohort of the Seven Countries Study, 3. Honolulu-Asia Aging Study, 4. Kaiser Permanent Medical Care Program of Northern California & 5. Framingham Study), four of them found a positive association between cholesterol levels and AD development, with only the Framingham study reporting no association (Kivipelto & Solomon, 2006). Interestingly however, the relationship between late-life serum cholesterol levels and dementia seems to suggest a negative association: out of 7 longitudinal studies (Göteborg, two from New York – Medicare beneficiaries, Washington Heights, Kuopio, Seattle & Rotterdam Study), five studies found a negative association between serum cholesterol levels and an AD diagnosis and only the latter two found no association (Kivipelto & Solomon, 2006).

In light of a possible interaction between cholesterol and Aβ production, there have also been several studies examining statins, or HMG-CoA reductase inhibitors, on AD pathogenesis. As statins are already commonly prescribed and very well tolerated, repurposing these cholesterol-lowering drugs for AD would streamline treatment and avoid the expensive and laborious process of drug development and approval. As of 2005, there have been four large retrospective studies on statins, two of which found that patients taking lovastatin, pravastatin or simvastatin had about a 70% lower risk of developing AD compared to age-matched controls (Jick, Zornberg, Jick, Seshadri, & Drachman, 2000; Wolozin, Kellman, Ruosseau, Celesia, & Siegel, 2000). Another study looking at postmenopausal women taking statins found that AD prevalence was decreased and that women with higher cholesterol levels were at higher risk of cognitive decline (Yaffe, Barrett-Connor, Lin, & Grady, 2002). The fourth study looked at use of statins in elderly individuals and found that there is a reduction in the risk for AD only in subjects younger than 80 years, with no effect seen in subjects 80 years and older (Rockwood et al., 2002). Although these studies suggest statins may reduce or delay AD progression,

subsequent meta-analyses have identified that many of these studies have failed to account for several factors including the "healthy user effect" (also known as "compliance bias") in which healthier patients are more likely to initiate and adhere to preventative therapies like consistently taking statins (Patrick et al., 2011). Other confounding factors include differing blood-brain barrier permeability among different statins being compared and the stage of AD in which statin usage was initiated (Shepardson, Shankar, & Selkoe, 2011). These criticisms have made a possible association between statin use and AD attenuation unclear.

Lipid Droplets

Lipid droplets (LDs) are spherical intracellular lipid storage organelles consisting of a hydrophobic core of cholesteryls and triacylglycerols enclosed by a phospholipid monolayer decorated with structural proteins, similar to lipoproteins (Penno, Hackenbroich, & Thiele, 2013). LDs bud off the endoplasmic reticulum and have dynamic contact sites with other organelles. As high amounts of free cellular lipids are toxic to cells, LDs serve as a means to sequester lipids for release during periods of stress and starvation (Farmer, Kluemper, & Johnson, 2019). Although these organelles were merely perceived as cytoplasmic fat inclusions, it is now known that LDs play a role in various metabolic diseases including diabetes (Greenberg et al., 2011). Recently, some studies have evaluated the role of abnormal LDs accumulations in the brains of individuals with neurodegenerative conditions; patients with Parkinson's and Alzheimer's disease have been found to have increased levels of LDs (citation?). Interestingly, in Alois Alzheimer's initial description of AD, he also commented on the presence of "adipose saccules" within glial cells (Stelzmann, Norman Schnitzlein, & Reed Murtagh, 1995), a characteristic that has been given little attention in the field of AD. Within the past few years, studies have suggested there may be an interaction between ApoE genotype and LD formation

(cit). When treated with hApoE4 astrocyte-conditioned media, wild-type fibroblasts produced more LDs than treatment with hApoE3 astrocyte-conditioned media (Area-Gomez et al., 2012). Similarly, induced pluripotent stem cells differentiated to astrocytes and expressing ApoE4 were found to have intracellular cholesterol accumulation compared to isogenic ApoE3 controls (Lin et al., 2018). Contrastingly, another study found that over-expressing ApoE2 and ApoE3 in the glia of *Drosophila melanogaster* resulted in LD accumulation while the ApoE4 variant did not (Liu, MacKenzie, Putluri, Maletić-Savatić, & Bellen, 2017).

Overview of ApoE/Lipoproteins in the central nervous system

Interestingly, the blood brain barrier does not allow for the transport of lipoproteins from the periphery into the central nervous system (CNS) (Wang & Eckel, 2014). As such, there exists distinct profiles of lipoproteins in the brain, completely different from what is seen in the periphery. Lipoproteins in the cerebrospinal fluid (CSF) were found to be either discoidal or spherical, largely resembling the size and density of plasma HDL (Wang & Eckel, 2014). Unlike in the plasma, ApoE is the most abundant lipoprotein in the CNS, followed by ApoJ and ApoA-I. In the brain, these apolipoproteins are predominantly expressed by astrocytes and microglia (Vance & Hayashi, 2010). Whilst ApoJ-containing lipoproteins are speculated to play a role in Aβ clearance, these particles normally contain the least amount of lipid. ApoE-containing lipoproteins in the CNS are generally the largest and the primary carriers of cholesterol. Once ApoE is expressed by astrocytes, astrocytes and other cell types will load ApoE with cholesterol and phospholipids via the ABCA1 transporter. These lipoproteins will then travel throughout the CNS where they either continue to pick up cholesterol from other cells or become uptaken by neurons via the LDL receptor (LDLR) superfamily to facilitate neuronal growth and development (Wang & Eckel, 2014).

The brain is the most cholesterol-rich organ in the human body, comprising approximately 20% of the body's total cholesterol, most of which is unesterified and found in myelin sheaths. Cholesterol is important not only for the development of myelin, but also for dendrite and axon formation in neurons as they require large amounts of membrane surface for these extensions (J. Zhang & Liu, 2015). As the brain cannot take up lipoproteins from the plasma, most of the cholesterol in the brain is supplied by *de novo* synthesis from the endoplasmic reticulum by astrocytes and to a lesser extent, neurons (J. Zhang & Liu, 2015).

Certain neurons in the brain including the pyramidal cells of the cortex and the Purkinje cells of the cerebellum express enzymes like cholesterol 24-hydroxylase, an enzyme capable of converting cholesterol to 24-hydroxycholesterol via hydroxylation (J. Zhang & Liu, 2015). Interestingly, oxysterols are capable of crossing the blood brain barrier. Thus, oxysterols are the main excretion pathway for cholesterol from the brain into the periphery (Björkhem et al., 1998). 24-hydroxycholesterol also activates certain nuclear transcription factors including Liver X receptors α and β which in turn upregulate expression of transport proteins involved in cholesterol efflux including ABCA1. Therefore it is believed that conversion of cholesterol to 24-hydroxycholesterol takes place when cells reach high cholesterol levels as a means to maintain homeostasis (Vance & Hayashi, 2010; J. Zhang & Liu, 2015).

Proposed role of apolipoprotein E4 in Alzheimer disease pathogenesis

Human ApoE is an apolipoprotein consisting of 299 amino acids with an N-terminal domain containing four α-helices 20-30 amino acids long and C-terminal domain forming one α-helix ~70 amino acids long. These two domains contain the receptor-binding region and lipid-binding regions, respectively (Chetty, Mayne, Lund-Katz, Englander, & Phillips, 2017). In humans, there exist 3 possible isoforms for this protein: ApoE2, ApoE3, and ApoE4. These three

isoforms are differentiated only based on two single nucleotide polymorphisms (SNPs), rs429358 and rs7412 varying amino acids at positions 112 and 158. The ApoE3 isoform, the most common variant, contains a thymine nucleobase at site +2985 resulting in a cysteine amino acid at position 112 and cytosine nucleobase at position +3123 resulting in an arginine at position 158 (Chaudhary, Kaushik, Ahmed, Kukreti, & Kukreti, 2018). The ApoE2 variant differs from ApoE3 by SNP rs7412 such that both the +3123 and +2985 positions contains a thymine and as a result both amino acids at positions 112 and 158 are cysteines. The ApoE ε4 variant differs from ApoE3 by SNP rs429358 which results in a cytosine at position +2985 such that both amino acids at positions 112 and 158 are arginine (Chaudhary et al., 2018; L. Zhong et al., 2016). These SNPs confer large changes in structure and function of these apolipoproteins, particularly between ApoE3 and ApoE4.

In 1993, Corder *et al* and Poirier *et al* independently determined ApoE ε4 as the strongest genetic risk factor for LOAD, and a year later, Chartier-Harlin *et al* identified ApoE ε2 as protective (Chartier-Harlin et al., 1994; Corder et al., 1993; Poirier et al., 1993). However, its mechanistic role in the disease remains hardly understood. ApoE is most highly expressed in the liver, followed by the brain (Holtzman, Herz, & Bu, 2012). In the central nervous system, ApoE plays a particularly important role as the primary cholesterol carrier, distributing lipids to cells to support re-myelination, proliferation and membrane repair (Verghese, Castellano, & Holtzman, 2011). ApoE ε3 is the most frequent ApoE allele in all populations, whereas the ApoE ε4 and ApoE ε2 alleles have frequencies ranging from 5-35% and 1-5%, respectively (Verghese et al., 2011). Although carrying an ApoE ε4 allele is not sufficient for AD development nor would it be considered a pathognomonic marker, having two copies of the ApoE ε4 allele increases risk for LOAD development by approximately 12-fold and shifts age of onset by approximately 2

decades compared to non-carriers. Indeed, the ApoE ε4 allele is present in 50% of all patients who develop LOAD compared to 20-25% in controls (Alonso Vilatela, López-López, & Yescas-Gómez, 2012; Verghese et al., 2011). While the role of ApoE ε4 in LOAD predisposition and onset is established, whether ApoE isoform associates with the rate of AD progression and cognitive decline post-onset remains controversial.

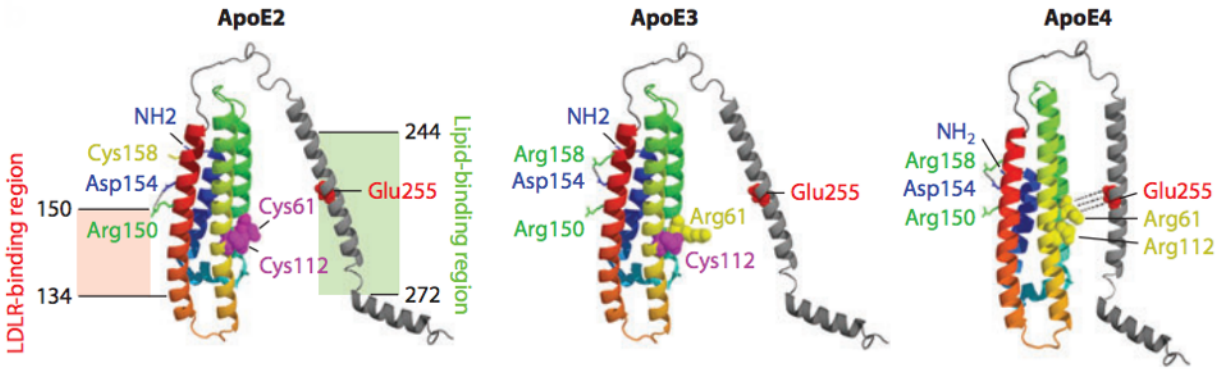


Figure 1.7: ApoE2, ApoE3, ApoE4 structure based on X-ray crystallography of lipid-free mouse and human ApoE. Structure of C-terminal domain (grey) is unknown, but modelled as helices based on circular dichroism spectroscopy and structure prediction software. Figure modified from Zhong et al. (N. Zhong & Weisgraber, 2009)

Partial lipid-free structural models suggest that ApoE3 and ApoE4 are structurally different from ApoE2 in that the E3/E4 isoforms can form salt bridges between Arg158 and Asp154 whereas in the E2 isoform, an alternative salt bridge is formed between Arg150 and Asp154 within the LDLR-binding region (Figure 1.7). Between E3 and E4, presence of an arginine in position 112 is believed to orientate Arg61 such that it can interact with Glu255, resulting in an interaction between the N and C domains (J.-T. Yu, Tan, & Hardy, 2014). Another hypothesis as to how the SNP differentiating E3 and E4 confers a structural difference is based on changes to helix 4 of the N-terminus. This theory posits the arginine/cysteine change in ApoE3 at position 112 affects the side chain position of arg114 resulting in a perturbation in the ionization of His140. This perturbation is thought to alter the charge distribution of helix 4,

which contains the LDL receptor-binding site, resulting in a structural change (Frieden & Garai, 2012).

Although the ApoE isoforms are structurally similar, *in vivo* structural diversions at these critical residues have profound effects on the apolipoprotein in terms of stability and receptor interactions which results in an overall impact on the lipoprotein and its metabolic dynamics. Separation of human CSF using gradient ultracentrifugation revealed that homozygous ε4 individuals have the highest level of lipid-depleted ApoE whereas homozygous ε2 had the lowest level (Hanson et al., 2013). ApoE4 lipoprotein particles also appear to be significantly smaller than ε3 and ε2 particles using non-denaturing gel electrophoresis (Heinsinger, Gachechiladze, & Rebeck, 2016). This can be explained as E3 particles appear to be much better acceptors of cholesterol than ε4 particles, inducing efflux approximately 3-4 times more efficiently than E4 (Heinsinger et al., 2016). In spite of these differences in lipidation and size, *in vitro* studies using E3 and E4 lipoproteins does not appear to alter cholesterol content in treated cells (Ye et al., 2005). However, ApoE isoform-dependent effects on Aβ generation can be seen in these same *in vitro* models. Other studies on APP trafficking investigating whether ApoE directly modulates secretase activities by altering cholesterol metabolism have found that ApoE may interact with LDL receptor-related protein-1 (LRP1), a plasma membrane receptor involved in receptor-mediated endocytosis. This interaction was found to stimulate an interaction between LRP1 and APP in an ApoE isoform-dependent manner such that more APP from the plasma membrane is recycled and therefore more Aβ is produced (Ye et al., 2005).

Other studies looking at ApoE in the context of Aβ have looked at aggregation and deposition. During Aβ aggregation and plaque development, Aβ monomers in the soluble phase change conformation into a beta-sheet structure and form nuclei. These beta-sheet structures then

become the "seed" which accelerates the process of fibrillogenesis, resulting in insoluble fibrils which can bind existing aggregates and become amyloid plaques. As ApoE prefers to bind to Aβ whilst in its β-sheet structure, therefore preventing "seeding", whether ApoE facilitates or inhibits Aβ aggregation is controversial. It has been shown that although ApoE3 interacts with Aβ more than ε4 which may be conducive to inhibiting fibril formation, ApoE4 is more likely to promote Aβ aggregation, to increase the level of and stabilize oligomers compared to E3 (Kanekiyo, Xu, & Bu, 2014).

ApoE also is known to play a large role in amyloid clearance as it is a known chaperone (Bu, 2009). There are several studies looking at ApoE-mediated clearance pathways for Aβ including clearance via microglia, astrocytes, transcytosis-mediated clearance across the blood-brain barrier, clearance through enzymatic degradation, as well as clearance via interstitial or perivascular fluid drainage (J.-T. Yu et al., 2014). In all of these instances, it appears that clearance mechanisms function in an isoform-dependent manner such that ApoE2 is the most efficient at clearance while E4 is the least (Vuletic et al., 2005). Other amyloid-independent roles for ApoE in AD progression and pathology explore its impact on tau pathology, neuroinflammation, general lipid metabolism and transport, metabolic alterations in the brain, mitochondrial dysfunction, and blood brain barrier permeability. To date, E4 is deleterious compared to E3 in all instances (J.-T. Yu et al., 2014).

The cholesteryl ester transfer protein

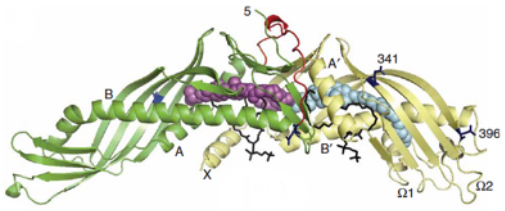


Figure 1.8: Crystal structure of CETP. Reprinted by permission from: Nature, Nature Structural and Molecular Biology, Crystal structure of cholesteryl ester transfer protein reveals a long tunnel and four bound lipid molecules, Xiayang Qiu, Anil Mistry, Mark J Ammirati, Boris A Chrunyk, Ronald W Clark et al., 2007

The cholesteryl ester transfer protein (CETP) is a plasma glycoprotein that catalyzes the transfer of neutral lipids between lipoprotein particles (Qiu et al., 2007). CETP was first described in 1965 by Nichols and Smith, and biochemically characterized 13 years later by Pattnaik NM *et al* (Nichols & Smith, 1965; Pattnaik, Montes, Hughes, & Zilversmit, 1978). CETP is a ~55 kDa boomerang-shaped protein with four structural units: An N- and C- terminal barrel at each end of the protein (each containing a beta-sheet and two alpha helices), a central β -sheet, and a C-terminal extension (Figure 1.8) (Masson, Jiang, Lagrost, & Tall, 2009). This structure confers a 60-Å-long hydrophobic tunnel and two openings that can be filled with cholesterol and plugged with phospholipids when not bound to lipoproteins (Qiu et al., 2007). There are several theories as to how CETP mechanistically transfers lipids including a carrier-mediated shuttle hypothesis in which it binds to one lipoprotein particle at a time and performs a neutral lipid exchange (Qiu et al., 2007) after traveling through the aqueous phase (e.g. plasma). Recent studies however are more in support of the tunnel hypothesis in which CETP bridges two lipoproteins and allows lipids to flow between the two (M. Zhang et al., 2018). CETP performs a heteroexchange wherein cholesteryl esters from HDL particles are transferred to LDL or VLDL particles in return for triglyceride particles (Paromov & Morton, 2003); there is no other transfer protein in human plasma that transfers these neutral lipids between lipoproteins (K. Kawano, Qin, Lin, Tall, & Jiang, 2000). As CETP's net transfer activity results in increased cholesteryl ester concentrations in pro-atherogenic LDL particles, CETP inhibitors are of great interest in the field of cardiovascular health as increased LDL cholesterol levels are an established modifiable

predictor for adverse cardiovascular events (Fernandez-Friera et al., 2017). CETP is predominantly expressed by the liver such that it is secreted into the plasma, however peripheral tissues including the spleen and adipose tissue may also contribute to plasma levels (Haas & Staels, 2015).

Interestingly, CETP has also been found to be expressed in the brain by astrocytes (Yamada et al., 1995); CETP concentrations in CSF were found to be about 12 percent of what is found in the plasma, whereas albumin concentrations are only about 0.6% of that in plasma suggesting CETP production in the brain (Haas & Staels, 2015).

CETP Polymorphisms and AD

In 1991, Inazu *et al*. published a paper in *The New England Journal of Medicine* linking a CETP deficiency caused by a splicing defect found in 5 Japanese families to markedly increased HDL levels, decreased LDL cholesterol levels, and a trend towards an increased lifespan (Inazu et al., 1990). This finding was further elaborated in 2003 by Barzilai *et al*. when they discovered in a cohort of 213 Ashkenazi Jewish individuals with exceptional longevity (mean lifespan of 98 years), and a unique lipoprotein phenotype with larger HDL and LDL particle sizes compared to age-matched controls (Barzilai et al., 2003). Interestingly this phenotype also associated with increased homozygosity for the I405V variant in CETP, a polymorphism that has been shown to reduce CETP levels (Bustami et al., 2016). Three years later in a follow-up study, this group reported that in an independent cohort of Ashkenazi centenarians, homozygosity in this polymorphism was approximately five-fold higher in non-demented subjects compared to demented Ashkenazi subjects aged 75-85. Further scores on the mini-mental state examination above 25 (on a scale of 30 in which a score from 24-30 is indicative of no cognitive impairment) were twice as likely in subjects homozygous for the CETPI405V genotype compared to control

subjects (Assoication, n.d.; Barzilai, Atzmon, Derby, Bauman, & Lipton, 2006). It was discovered that the CETPI405V polymorphism was also associated with a lower rate of memory decline and a decreased risk of incident dementia (Chen, Li, Zou, & Fu, 2014; Sanders et al., 2010; L. Yu et al., 2012).

Given that CETP directly modulates lipids within lipoprotein classes, that CETP it is expressed in the brain, and that lipoprotein profiles and cholesterol metabolism are so strongly associated with cognitive function, multiple studies have thus performed gene-gene interaction studies on CETP and ApoE. Results, for the most part, suggest an interesting correlation between both. Murphy *et al*. determined that in a cohort of 318 elderly individuals with AD or mild cognitive impairment, polymorphisms in the CETP promoter that decrease expression levels and increase HDL levels (CETPI405V and CETPC629A) appeared to be protective in ApoE ε4 carriers, however absence of these polymorphisms appear to be protective in ApoE ε4 non-carriers (E. A. Murphy et al., 2012). This finding was also supported by Rodríguez *et al* who found that in a sample of 286 AD subjects, homozygosity for CETPC629A allele resulted in a three times lower risk of developing AD compared to cysteine carriers, however the CETPI405V allele had no effect on AD risk (Rodríguez et al., 2006). Arias-Vasquez *et al*. also confirmed some of these findings: in a cohort of 544 AD cases, risk of AD for non-carriers of the ApoE ε4 allele was 1.67-fold higher for individuals homozygous for the CETPI405V SNP, however no significant effect was seen on AD risk for ApoE ε4 carriers (Arias-Vásquez et al., 2007). Finally, this effect was corroborated by Saunderman *et al*.: in a cohort of 909 non-demented subjects that were annually assessed under the Einstein Aging study, the valine allele of the CETPI405V was advantageous in ApoE ε4 carriers and associated with a higher risk of dementia in non-carriers (Sundermann et al., 2016). However, other reports such as Qureischie *et al*. found that in a

cohort of 351AD patients, there was no association between the two aforementioned CETP haplotypes and AD risk (Qureischie et al., 2008). As well, Johnson *et al* found no significant associations of CETP genotype with cognitive function with age (W. Johnson et al., 2007).

Limitations of WT mice as a model for human lipid metabolism

Although the role that cholesterol plays in AD is not completely elucidated, it is irrefutable that circulating lipids play a key role in disease pathogenesis; this is evident not only based on epidemiological data and established co-morbidities, but most notably the APOE gene having the greatest known effect on risk of developing LOAD (Kanekiyo et al., 2014). Over the past decade of research however, most of the field of AD using mice as a biological model have overlooked or perhaps underestimated the differences between the lipid metabolism profiles of humans and rodents. Indeed, with mice being the most commonly used vertebrates for *in vivo* modelling of the disease (Elder, Gama Sosa, & De Gasperi, 2010), it is imperative to address discrepancies between their physiological systems and that of demented persons, particularly a metabolic system so clearly intertwined with the disease being modelled.

Unfortunately, the mouse lipoproteome remains highly uncharacterized, even more so for that of the CNS. This is particularly evident with the lack of literature on murine "minor" lipoprotein-associated proteins (where the "major" apolipoproteins include ApoB, ApoA, ApoE, etc.) which are known to play important roles in transport, coagulation, and even glucose metabolism in humans (Gordon et al., 2015). Indeed, over 90 "minor" HDL-associated proteins were found in human plasma HDL distributed across a diverse collection of particles in patterns based on particle density, size and charge, creating sub-families. These sub-families are thought to confer unique functions and roles for lipoproteins within their broader classes; the same diversity can be said for ApoB-containing lipoprotein particles like LDL wherein there have

been over 20 minor proteins identified with functions in inflammation, lipid hydrolysis and lipid transport (Gordon et al., 2015). Nevertheless, there have been some studies looking at more salient differences between lipoprotein classes in mouse serum versus human serum. Firstly, murine serum, unlike human serum contains only very low levels of ApoB and ApoB-containing lipoproteins (Gordon et al., 2015; Steenbergen et al., 2010). As a result, while humans have only about 20-30% of their total serum cholesterol in HDL particles, about 80% of serum cholesterol is found in the HDL fraction of mice, making them resistant to the development of atherosclerotic lesions on a standard diet (Grass et al., 1995). Some laboratories have found that it is possible to induce atherosclerosis in some strains of mice using very fatty diets (15% fat, 1.25% cholesterol): out of 16 inbred mouse strains tested on this diet for 14 weeks, 4 strains were very susceptible to lesion formation (including the commonly used C57BL/6), 6 had low to moderate susceptibility, and 6 remained resistant (Paigen, Ishida, Verstuyft, Winters, & Albee, 1990). This is noteworthy as these studies have discerned that the development of these atherosclerotic lesions necessarily requires both a reduction of HDL cholesterol and an elevation of VLDL/LDL cholesterol in the serum of these mice, which is a profile more aligned with that of a normolipidemic human (Nishina, Verstuyft, & Paigen, 1990). Although studies did indeed find that susceptible strains of mice fed an atherogenic diet had an approximately 50% increase in VLDL/LDL cholesterol levels, 1.5- to 2-fold increase in plasma ApoE, and an increase of ApoB, the ApoB-increase was due to increases in ApoB-48 as opposed to ApoB-100 (Nishina et al., 1990). While ApoB-48 is a truncated form of ApoB-100 produced in the intestine, only chylomicrons carry ApoB-48 while VLDL/LDL particles carry the 100-form, the only form of ApoB recognizable by LDL receptors (Nishina et al., 1990; Olofsson & Borèn, 2005). Furthermore, mice on these diets were also prone to fatty livers and gallstones as early as 3

weeks into the diet, making them ill-suited as models of healthy individuals (Nishina et al., 1990).

To attenuate the differences between murine lipoproteome and that of humans, several labs have generated mice over-expressing human ApoB-100 (hApoB-100). These mice had hApoB-100 in the LDL fraction, ApoB-100 levels similar to the levels seen in normolipidemic humans and increased LDL cholesterol levels compared to non-transgenic controls. However, about 30-40% of serum cholesterol and 60% of serum triglycerides was contained in LDL particles whereas humans normally have about 60% and 30%, respectively (Callow, Stoltzfus, Lawn, & Rubin, 1994; Grass et al., 1995; Linton et al., 1993).

The primary reason as to why mice have a serum cholesterol distribution different from that of humans is due to the fact that mice lack CETP (Gordon et al., 2015; Grass et al., 1995; K. Kawano et al., 2000). Interestingly, transgenic mice expressing CETP alone were found to have a redistribution of serum cholesterol from HDL to VLDL/LDL, increasing LDL/VLDL cholesterol levels and lowering HDL cholesterol levels (Grass et al., 1995). Although CETP expression alone does not bring LDL cholesterol levels to 60% as seen in humans, it is increased 2-fold compared to non-transgenic littermates and triglyceride lipoprotein distribution is almost identical to what is seen in humans compared to hApoB-100 or CETP and hApoB-100 double-transgenic mice where triglyceride levels in LDL particles are too high (Grass et al., 1995). As such, although imperfect, CETP transgenic mice are very good models for the human lipoproteome and should be used as a baseline model for studies in diseases tied to lipid metabolism, including AD.

Preliminary data

To simulate *in vivo* human physiology in in terms of lipid metabolism in AD mouse models, our lab crossbred a CETP transgenic mouse strain to APP transgenic strains (McGill-Thy1-APP Tg mice which have hAPP KM670/671NL (Swedish) and V717F (Indiana) mutations. We observed a 5-fold increase in insoluble Aβ levels in the brains of CETP/APP double transgenic mice compared to APP transgenic mice.

Hypothesis/Research question

The goal of this thesis is to discern whether this effect of increased Aβ levels is caused by CETP expressed in the periphery or by the 9-fold lower levels of CETP expressed in the brain, and whether these effects are modulated by the ApoE isoform. As the ApoE ε4 allele is associated with increased AD risk and CETP/APP double transgenic mice have increased levels of Aβ, and studies on CETP SNPs have discerned that CETP-lowering polymorphisms are protective in ApoE ε4 carriers, I hypothesized that CETP-mediated Aβ increase is enhanced in the presence of ApoE4 relative to ApoE3, and that this effect will be lost with addition of a CETP inhibitor. As ApoE4 constitutes the greatest genetic risk for sporadic AD, its potential influence on CETP-mediated Aβ increase was analyzed using immortalized mouse astrocytes provided by Dr. David Holtzman's laboratory from Washington University. These astrocytic cell lines express either human ApoE3, or ApoE4 and were utilized for various experiments. First, I characterized these astrocytes in terms of lipid droplet deposition and found that the ApoE4 astrocytes had a much higher level of lipid droplets compared to ApoE3. This finding was corroborated by a paper recently published using the same cell lines (Farmer et al., 2019). Secondly, using concentrated conditioned media from these astrocytes, I identified lipoprotein fractions using size-exclusion chromatography. I

incubated this concentrated media with and without exogenous recombinant CETP (rCETP) and with and without CETP inhibitor (evacetrapib) and assessed modulations that occurred with the presence of and inhibition of rCETP activity. I next developed CETP stably-expressing hApoE astrocytes and co-cultured these astrocytes and APP over-expressing SH-SY5Y cells. I also co-cultured wild-type hApoE astrocytes with and without titrated amounts of rCETP and/or CETP inhibitor. The media from these co-culture assays were assessed for CETP activity and Aβ40 was quantified by enzyme-linked immunosorbent assay (ELISA).

A limitation of the CETP transgenic (CETP tg) mice is that they express CETP in the periphery as well as in the brain. I have therefore generated an adeno-associated viruses (AAVs) with liver-specific promoters to restrict the expression of CETP to the periphery. The five viruses that have been developed are: mock (GFP), wild-type CETP as well as 3 transfer mutants. *In vivo* assessment of the wild-type CETP virus has ensured that the CETP is secreted and active, based on commercial CETP activity assays.

Materials and Methods

Site-Directed Mutagenesis: CETP AAV plasmid obtained from Daniel J. Rader under a material transfer agreement contained a human wild-type CETP insert driven by the liver-specific human thyroglobulin binding globulin (TGB) promoter followed by a SV-40 poly-A tail. This plasmid was mutated to produce three variants of CETP tunnel mutants: V198W, Q199A, T155Y using site-directed mutagenesis (SDM). Primers were designed as follows using QuikChange Primer Design software by Agilent:

V198W F: GCTGGCAGCCCTTGTCTGCCAAAAATCGGCCATGATGTTAGA,

R: TCTAACATCATGGCCGATTTTTGGCAGACAAGGGCTGCCAGC;

Q199A F: GCTGGCAGCCCTTGTCGCGACAAAATCGGCCAT

R: ATGGCCGATTTTGTCGCGACAAGGGCTGCCAGC;

T155Y F: CAGTCAGGGGCATCGTACCGCACTCTACCAGA,

R: TCTGGTAGAGTGCGGTACGATGCCCCTGACTG;

SEQ F: TACCCAGATATCACGGGCGA;

R: GACTCGCTCAGAGAACCAGA

SDM was preformed using PfuUltra High-Fidelity DNA Polymerase I protocol and components by Agilent (Catalog #600380). Polymerase chain reaction (PCR) protocol as follows: 95°C 30 seconds, 95°C 30 seconds, 55°C 1 minute, 68°C 6 mins with cycles 2-4 repeated 18 times. 40 µl of PCR product (from total reaction volume of 50 µl) was digested with 1 µl DpnI enzyme and incubated at 37°C for 2 h.

Plasmid preparation: wild-type pAAV2/8, pHelper, CETP, pHelper (Cell Biolabs, Catalog #340202), GFP (Cell Biolabs, Catalog #AAV-400), and mutant CETP plasmids from SDM were transformed into NEB® 5-alpha Competent E. coli (High Efficiency- Catalog

#C2987) according to NEB transformation protocol. 5 colonies per plasmid picked after overnight culture on ampicillin agar plates and inoculated into small LB liquid cultures for approximately 16 h. Using NucleoSpin® Plasmid kit (Machery & Nagel Catalog #740588), midi preps preformed using cultures according to Machery & Nagel protocol and colonies with the correctly mutated sequences were determined by Sanger Sequencing at the McGill University and Génome Québec Innovation Centre using "SEQ" primers listed for CETP plasmids, M13 primers provided by Genome Quebec for pAAV2/8, and restriction enzyme digest by BamHI for pHelper plasmid validation. Desired colonies were re-inoculated into larger overnight cultures of liquid LB media with ampicillin. Liquid cultures were pelleted the next day and midi preps were preformed using NucleoBond® Xtra Midi kit (Machery & Nagel Catalog #740410). DNA plasmid concentration determined using BioTek Take3 Micro-Volume Plate and Gen5 software.

Adeno-associated virus production: AAV was produced using a protocol modified from CellBiolabs "AAV Helper Free Packaging System" using a chimeric packaging construct pAAV2/8 wherein the rep gene of AAV2 was fused to the cap gene of AAV8, producing an AAV with a serotype 8 capsid. The day before transfection, 6 plates of HEK293-T cells were seeded on 10-cm dishes to be about 70-80% confluent the next day. Triple-transfection of pHelper, pAAV-RC2/8, and pAAV of interest (i.e. pGFP or pCETP) with a DNA ratio of 1.5:1.2: 1 respectively done using a 4:1 ratio of polyethylenemine (PEI, μg): total DNA (μg). Cells were harvested after 72 h incubation using PBS and pellet re-suspended in AAV lysis buffer. Cell pellet underwent 3 freeze-thaw cycles using a dry ice/ethanol bath and a 37°C water bath and Benzonase added to cell lysate followed by a 30-minute incubation at 37°C. Lysate was centrifuged at 4500 rpm for 30 min at 4°C and vector-containing supernatant was loaded on an iodixonol density gradient prepared using 60%, 40%, 25%, 15% Iodixanol, NaCl/PBS-MK and

PBS-MK. PBS-MK buffer was made using 0.0027 M $MgCl_2$, 0.002 M KCl, in 1x PBS. NaCl/PBS-MK was made with all of the components of PBS-MK, with the addition of 1 M NaCl. All buffers were sterile filtered through a 0.22-μm filter and stored at 4°C until use. Density gradient was ultracentrifuged at 63,000 rpm for 2.5 h at 4°C and vector was collected from the 40% iodixanol layer. Vector was concentrated using Ultra-15 10K Centrifugal Filter Devices (Amicon, Catalog #UFC901008), filtered with a 13 mm 0.2 μm syringe filter (Millipore, Catalog #SLLG013SL), aliquoted in PCR tubes, and stored at -80°C until required. Titer determination was done by qPCR using SsoAdvanced SYBR green supermix (Biorad, Catalog # 1725270) and Biorad CFX384Touch cycler. Primers used were as follows:

ITR F: GGAACCCCTAGTGATGGAGTTG,
R: GCGTCGGGCGACCTTTG;

SV40 F: GACAAACCACAACTAGAATGCAGTG,
R: CATCTCCCCCTGAACCTGAAAC;

EMBL8 F: AGATCAGTTGGGTGCACGAG,
R: TTCATTCAGCTCCGGTTCCC;

Results were analyzed using CFX Maestro software (Biorad). All primers were ordered from Integrated DNA Technologies. Titer was determined using standards made from pure plasmid DNA based on Addgene "AAV Titration by qPCR Using SYBR Green Technology" protocol (Addgene, 2016).

Maintenance: Immortalized mouse astrocytes, provided as a gift from David Holtzman's laboratory, were cultivated in 1X Dulbecco's modified Eagle medium (DMEM – Wisent, Catalog # 319-005-CL) containing 4.5 g/l glucose, 0.584 g/l L-glutamine and 0.11 g/l sodium pyruvate was supplemented with +10 % v\v Fetal Bovine Serum (FBS, Gibco, Catalog

#16000044), 0.2 mg/mL Geneticin (Gibco, Catalog # 10092772) and 1.0 mM Na/pyruvate (Gibco, Catalog # 11360070). hApoE-expressing astrocytes were verified as having correct genotype through genomic DNA genotyping at Génome Québec Innovation Centre (TaqMan® genotyping of SNPS rs429358 & rs7412 of gDNA using the PureLink™ Genomic DNA Mini Kit, Invitrogen, Catalog # K182001). SH-SY5Y cells stably over-expressing APP were cultivated in 1X DMEM (Wisent, Catalog # 11360070) sodium pyruvate supplemented with 10% v\v Fetal Bovine Serum (FBS, Gibco, Catalog # 16000044), 250 µM Hygromycin B (Invitrogen, Catalog #10687010) and 2 mM L-glutamine. HEK293-T cells were grown using DMEM (Wisent, Catalog # 319-005-CL) containing 4.5 g/l glucose, 0.584 g/l L-glutamine and 0.11 g/l sodium pyruvate was supplemented with +10% v\v Fetal Bovine Serum (FBS, Gibco, Catalog #16000044) while HepG2 cells were cultivated with alpha-minimum essential medium (Lonza, Catalog #12-169F).

Co-cultures: SH-SY5Y cells were seeded in 6-well plates at 4.0 x 10^5 cells per well, followed by co-seeding of ApoE3 or ApoE4 astrocytes at 2.0 x 10^5 cells per well of using co-culture media composed of DMEM – Wisent, Catalog # 319-005-CL) containing 4.5 g/l glucose, 0.584 g/l L-glutamine and 0.11 g/l sodium pyruvate was supplemented with +10% v\v Fetal Bovine Serum, 1.0 mM Na/pyruvate (Gibco, Catalog # 11360070), and 2 mM L-glutamine. Cell types were also seeded alone at the same cell counts per well. 24 h after seeding, media was replaced with serum-free DMEM supplemented with either PBS-suspended bovine serum albumin (BSA, Sigma) or rCETP (Roar biomedical, Catalog # SMB00603-200UL) with and without evacetrapib. Cells were incubated for an additional 24 h, media and lysates were collected for ELISA and western.

Transfections: To create CETP stable ApoE3 or ApoE4 astrocytes, transfections were done in 10cm dishes at approximately 70% confluency using FuGene HD (Promega, Catalog # E2311) at a 2:1 ratio of FuGene reagent and 10 μg DNA. Cells were maintained in astrocyte media with increasing amounts of Hygromycin B (Invitrogen, Catalog # 10687010) until a final concentration of 600 μg/ml. Stable cells were maintained using astrocyte media supplemented with 250 μg/ml Hygromycin B. HepG2 transfections were preformed using Lipofectamine 2000 (Invitrogen, Catalogue # 11668) in a 12-well format using a 2:1 ratio of Lipofectamnie reagent and 2 μg DNA per well. $2x10^5$ cells were seeded per well and transfection was performed the next day by incubating DNA and Lipofectamine reagent in 500 μl Opti-MEM for 5 minutes, incubating these two mixtures combined for 30 minutes, and replacing the growth media of the HepG2 cells with this transfection complex for 6 h in the incubator. Fresh media was then replaced; cell culture supernatant was collected 48 h later and assessed for CETP protein and activity by western blot and CETP activity assay, respectively.

CETP activity: CETP activity was determined using a CETP activity assay kit (Roar biomedical, Catalogue # MAK106-1KT) 5 μL of cell culture supernatant or 1 μL of mouse serum was incubated with 0.3 μL donor and 0.3 μL acceptor reagents in a 35 μL total reaction volume, with and without 1μM evacetrapib to discern ΔCETP activiy. The reaction mix was incubated for 2 h at 37°C in a water bath and the fluorescence (λ_{ex} 465/λ_{em} 535) was measured.

Conditioned Media collection for FPLC: 15 10 cm dishes of ApoE3 and ApoE4 astrocytes were seeded and grown to confluency in 10% FBS astrocyte media. At complete confluency, media was replaced with serum-free DMEM conditioned media was collected after 72 h and stored for no longer than one week at 4°C. 150ml conditioned media of each cell type was spun down for 10 minutes at 800g/4°C and

subsequently concentrated approximately 75x using Ultra-15 10K Centrifugal Filter Devices (Amicon, Catalog #UFC901008) and aliquoted for treatments: 2mg/ml BSA (Sigma); 2mg/ml BSA with 0.5 μg/ml rCETP (Roar biomedical Catalog # SMB00603-200UL); 2mg/ml BSA with 0.5μg/ml rCETP and 5μM evacetrapib. BSA was added to all samples both to normalize 280nm FPLC spectrum and to stabilize CETP activity. Treated media was incubated for 18 h at 37°C and run on a Superose 6 Increase 10/300 GL column (GE life sciences) using an ÄKTAmicro (GE life sciences) at a flow rate of 0.4 ml/min using 0.02 M $NaPO_4$, 0.15 M NaCl, pH 7.4, 0.03% EDTA, and 0.02% sodium azide. 300 μl fractions were collected and analyzed within 48 h (stored at 4°C) using Infinity™ Triglyceride liquid stable reagent and (Thermo Fisher Scientific, Catalogue # TR22421) and Amplex Red Cholesterol Assay kit (Thermo Fisher Scientific, Catalogue # A12216). 280 nm protein chromatogram was generated and analyzed using Unicorn 5.31 (GE life sciences) software.

Animal housing: Wild-type mice of strain C57BL/6J wild-type strain were injected with 60 μl or 100 μl of CETP AAV via tail vein. Blood collection done through tail vein once a week, and serum was separated using Microvette® 100 LH tubes (Sarstedt, Catalog # 20.1282.100). CETP transgenic mice of strain B6.CBA-Tg(CETP)5203Tall/J (Jackson strain no.: 003904) and non-injected wild-type mice were used as controls, all mice were maintained on a low-fat diet enriched with 1% cholesterol (TD.140215). Mice were handled, housed and maintained per McGill University standard operating procedures.

Aβ40 ELISA: Aβ40 levels from cell culture supernatant was determined using sandwich-based ELISA using MaxiSorp 96-well clear plates, Anogen Aβ40 (Biotech Laboratories, MO-M40095B) capture antibody at a concentration of 1:15,000 in carbonate buffer and Anogen APP detection antibody biotinylated in-house at a concentration of 1:20,000 in

liquor buffer with 10% SEA BLOCK (Thermo Scientific, Catalogue #37527). After cell culture supernatant was spun down at 1000 g for 10 minutes, 50 µl media samples were loaded in duplicates. Detection was done using poly-SA HRP enzyme at a dilution of 1:20,000 and TMB substrate solution (Thermo, Catalogue # N301), which was stopped using 50 µL of 10% H_2SO_4 per well.

Lipid Droplet Staining: $8x10^4$ cells of ApoE3 or ApoE4 astrocytes were seeded onto Poly-D-Lysine/Laminin 12 mm pre-coated cover slips (Corning® BioCoat™ , Catalogue # 354087) on a 12-well plate. The following day, positive control cells were treated with an oleic acid-albumin complex (Sigma, Catalogue #O3008-5ML) such that media was brought to a concentration of 300 µM. 24 h later, cells were washed with PBS, fixed using formaldehyde solution 4%, buffered, pH 6.9 (Sigma, Catalogue # 1004965000) for 15 minutes, and stained with 20 µM BODIPY (Invitrogen, Catalogue #D3922) and 10 µM Hoechst 33342 (Invitrogen, Catalogue #H3570) in PBS for 10 minutes. Cells were then mounted on glass microscope slides using VECTASHIELD® Antifade Mounting Medium (VECTOR laboratories, Catalogue #H-1000), sealed with nail polish, and imaged using the Hamamatsu Monochrome camera on a Axiovert 3 fluorescence microscope at 40x objective (BODIPY: λ_{ex} 493/λ_{em} 503; Hoechst 33342: λ_{ex}350/λ_{em} 461).

Results

Lipid droplets in ApoE3/E4 Astrocytes

It has been shown that AD patients have elevated lipid accumulation in the form of LDs in both peripheral cells and neurons (Area-Gomez et al., 2012; Mandas et al., 2012; Pani et al., 2009). To discern whether there were differences in neutral lipid accumulation between ApoE astrocyte isoforms, cells were seeded on pre-coated cover slips and analyzed for LD

accumulation using the neutral lipid dye 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY 493/503) that fluoresces upon integration into lipid droplets. We found that there are increased levels of LDs in ApoE4 astrocytes relative to the ApoE3 astrocytes (Figure 2.1), which was also seen upon addition of oleic acid, a stimulator of LD formation (Rohwedder, Zhang, Rudge, & Wakelam, 2014).

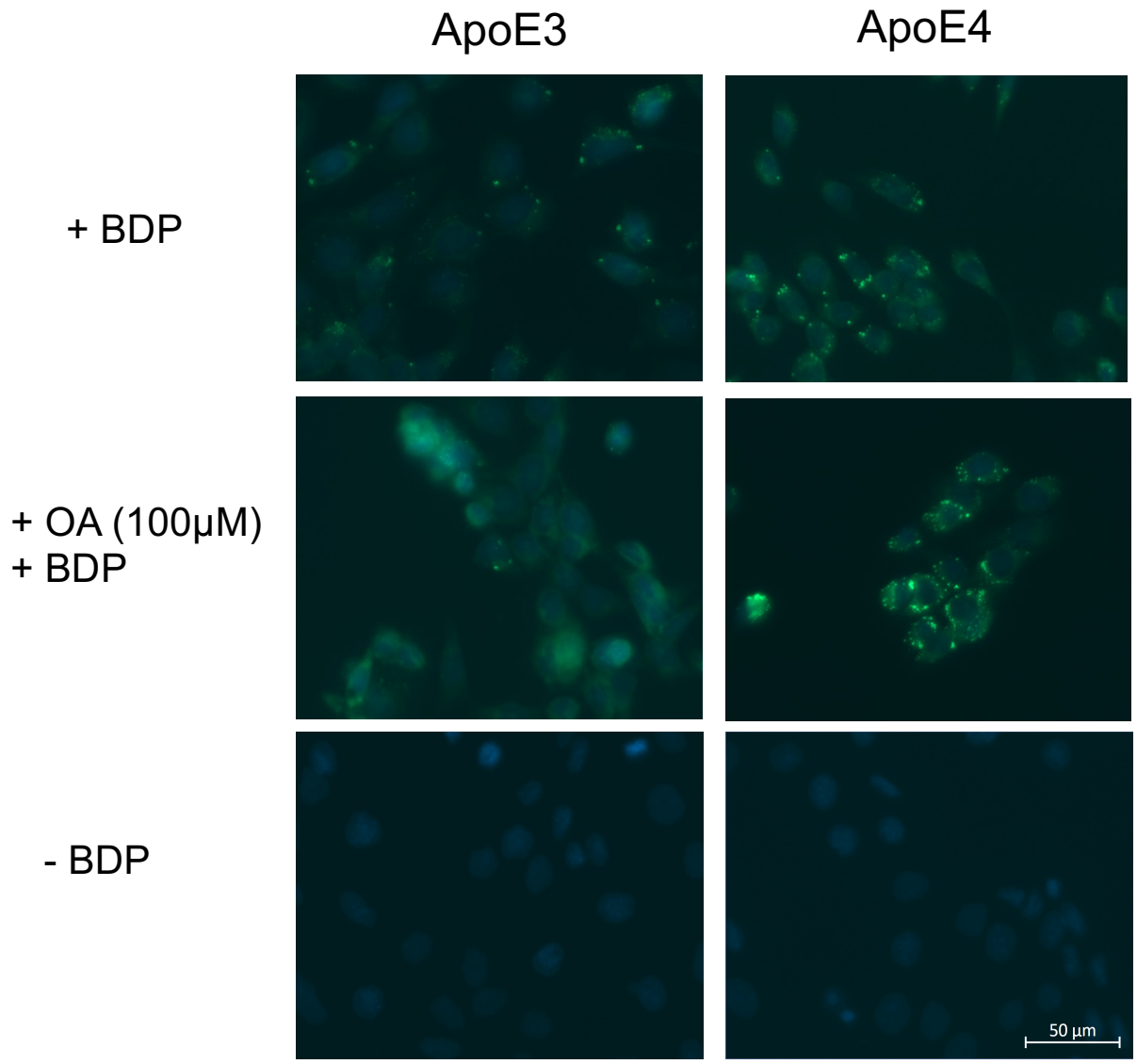


Figure 2.1: Marked differences in BODIPY lipid droplet staining in murine hApoE astrocytes. Images of fixed astrocytes treated with and without oleic acid (OE- 100 μM) for 24 h and stained with BODIPY 493/503 + (BDP) (green); Hoechst, and Hoechst alone (for nuclei, blue). All images exhibit BODIPY + Hoechst channel merge and cells at 40x magnification. Representative images from 3 biological replicates.

CETP activity modulates astrocytic lipoproteins

Within the context of the blood, it has been well established that CETP is capable of exerting lipid transfer between lipoprotein particles. However, to date it has not been investigated whether CETP is able exert similar transfer activity on lipoproteins of the CNS. To evaluate if CETP can modulate endogenous astrocyte-secreted lipoproteins, size-exclusion chromatography was preformed to evaluate the lipoprotein profiles under different treatments. These chromatograms are generated from a 280 nm UV-detector and so peaks correspond to distinct protein classes separated by size (largest to smallest from left to right, Figure 2.2). As lipoproteins are have high amounts of protein (i.e. the apolipoproteins that decorate the lipoprotein monolayer), lipoprotein peaks can be picked up through this readout. The column was first calibrated using commercial, purified human lipoproteins to develop a standard in terms of particle size: elution volume (Figure 2.2A). These lipoproteins (as well as BSA) were run individually to discern what particle or protein each peak corresponds to (data not shown), and then together to create one example (Figure 2.2A). hApoE3 and hAPoE4 conditioned media was collected, concentrated and aliquoted into samples with no treatment (albumin only), rCETP + albumin and rCETP + albumin + evacetrapib to assess CETP-dependent effects on the 280 nm profile of these lipoproteins. If a lipoprotein peak was, for example, shifted to the right with CETP treatment, one could infer that these lipoproteins were smaller due to CETP activity. It was found that after 16 h incubation, the rCETP was still active, and treatment groups with both rCETP and evacetrapib demonstrated inhibition of activity (Figure 2.2B). rCETP and evacetrapib

run alone on the size exclusion chromatogram did not result in any absorbance at 280 nm; rCETP protein concentration was too low to be picked up by the UV-detector and evacetrapib exists as a

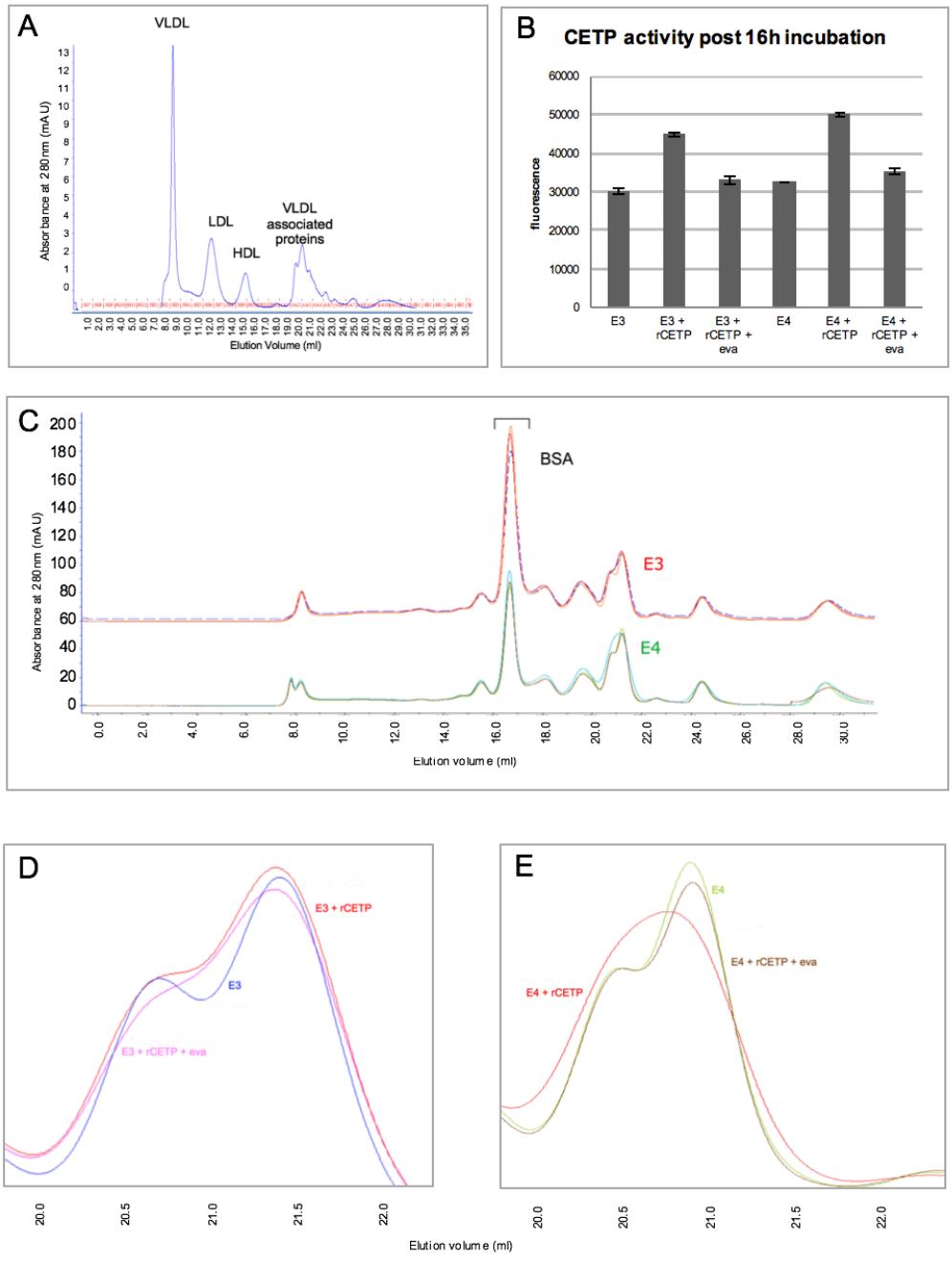


Figure 2

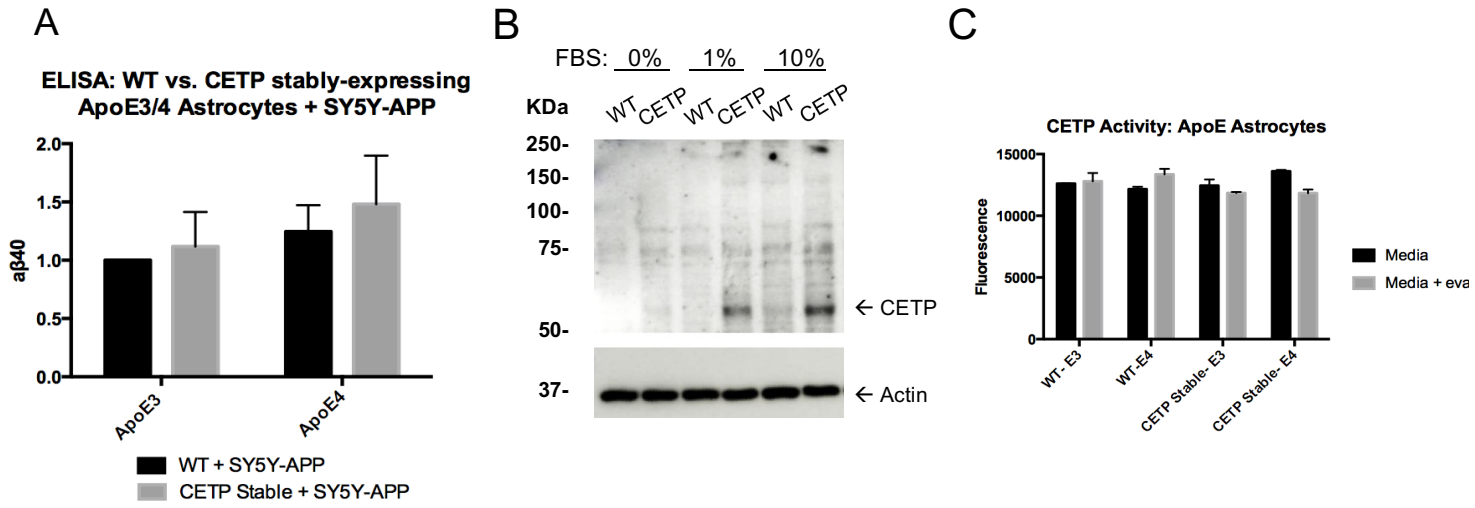
(**A**) Standardization of FPLC gel filtration chromatogram recorded at 280 nm using human VLDL, LDL, and HDL. (**B**) CETP activity from treated hApoE astrocyte supernatant aliquots post 16 h incubation using ~65x concentrated 48h conditioned serum-free media. (**C**) FPLC gel filtration chromatogram recorded at 280nm of hApoE3 and hApoE4 astrocyte supernatant treated with rCETP and incubated for 16 h. (**D-E**) Enlarged depiction of the 280 nm chromatogram between elution volume 20.0-22.0ml demonstrating rCETP-mediated lipoprotein peak modulation. Representative chromatograms of three independent biological replicates (n=3).

chemical compound with no peptide components (data not shown). The lipoprotein peaks were discerned based on previous literature using these same astrocytes (Fagan et al., 1999), as well as triglyceride levels only being present within fractions at elution volume of 20-22ml (data not shown). For both hApoE3 and hApoE4 lipoprotein conditioned media, the two lipoprotein peaks appeared to be somewhat homogenized with addition of rCETP such that the baseline "shoulder' disappears, however only in hApoE4 conditioned media was this effect reversed with addition of evacetrapib (Figure 2.2C-E).

Co-Culture system using CETP stably-expressing astrocytes

To discern if CETP can exert an effect on Aβ levels in an ApoE isoform-dependent manner, co-cultures were cultivated using APP-SH-SY5Y cells: neuroblastoma cells (representative of neurons) expressing additional exogenous APP, and hApoE murine astrocytes stably expressing CETP versus wild-type hApoE murine astrocytes. Aβ40 levels were then quantified by ELISA. Knowing that CETP increased Aβ levels in the double transgenic mouse model, we hypothesized that CETP alters the composition of astrocyte-derived lipoprotein particles taken up by neurons, which in turn confers an increase in Aβ production. Although the difference between Aβ40 levels for astrocytes expressing versus not expressing CETP was non-significant for both ApoE genotypes, a trend was seen towards increased Aβ40 with presence of CETP (Figure 2.3A). However, it was also evident that levels of CETP expression were very low by these astrocytes—levels of secreted protein were not detectable by western blot in the cell culture supernatant due the abundant levels of albumin, which runs at approximately the same height as CETP. Although CETP was detectable in the lysate in dependence of the FBS concentration (Figure 2.3B), CETP activity was not seen (Figure 2.3C), suggesting secreted

levels are quite low. As the CETP activity assay is not very sensitive, whether stable expression of CETP by hApoE astrocytes has an effect on Aβ40 levels is unclear.



Co-Culture system using exogenous recombinant CETP

Figure 2.3: Murine hApoE astrocytes stably express low amounts of CEPT. (**A**) Aβ40 ELISA data from 24 h co-cultures of APP-SH-SY5Y cells with CETP-hApoE murine astrocytes versus WT hApoE murine astrocytes. Normalized to ApoE3, (mean ± SEM, n = 3). (**B**) Western blot demonstrating lysates of CETP-hApoE murine astrocytes stably express CETP in dependence of FBS concentration. (n =2) (**C**) CETP activity assay using 24 h conditioned media from CETP-hApoE murine astrocytes. Normalized to WT ApoE3, (mean ± SEM, n = 2).

To circumvent the issue of low levels of secreted CETP protein with the stably expressing astrocytes and to control for any downstream metabolic differences between the astrocytes stably expressing CETP versus those that do not, exogenous recombinant CETP was used as a treatment in co-culture systems using wild-type hApoE astrocytes. APP-SH-SY5Y cells were co-seeded with hApoE murine astrocytes and after 24 h were treated with either rCETP or albumin with and without evacetrapib. 24 h post-addition of rCETP to media with lipoprotein-deficient serum (LPDS), CETP protein was detectable at high concentrations in the media (Figure 2.4A) with no changes to cell proliferation, ApoE levels or APP levels (Figure 2.4B). In accordance with literature on ApoE expression, there was a trend towards lower levels of secreted ApoE4

compared to ApoE3 protein levels (Farmer et al., 2019). As albumin runs approximately at the same height of CETP and often makes it difficult to visualize CETP by western blot, aliquots of CETP treated media pre-addition of LPDS was separately incubated in a 96-well plate at 37°C without the presence of cells. This media was also run on the western (Figure 2.4A) to visualize true baseline CETP levels post 24 h incubation. This does not, however, account for possible modifiers of CETP levels in co-cultures including secreted proteases or endocytosed protein which may decrease levels and high amounts of LPDS albumin which would stabilize levels, but is the best control we have. Interestingly, full-length APP (flAPP) levels in co-cultures were significantly higher than those in APP-SY5Y-alone treatments. As the antibody used for APP detection (22c11) only detected human APP from the APP-SH-SY5Y stable cells, the increase in APP levels seen here is either due to increased APP-SH-SY5Y proliferation as a result of an additional source of energy in the form of excess triacylglycerol, or increased APP production. Other studies have also found that co-culturing neuronal cells with glial cells results in a 3-4 increase in expression of APP whereas co-cultures with mouse embryonic fibroblasts (MEFs) do not. These studies also found that this increase in APP expression could be rescued with addition of both recombinant ApoE and recombinant cholesterol-free ApoE, arguing for a possible direct receptor action (Huang, Zhou, Wernig, & Südhof, 2017). In addition, CETP activity levels were relatively high with 0.4 μg /ml rCETP and still detectable at 0.04 μg/ml rCETP (Figure 2.4D) post-incubation. hApoE4 astrocytes produced higher Aβ40 levels than ApoE3 astrocytes demonstrating an ApoE isoform-dependent effect in co-cultures. However, no CETP activity-dependent effect was seen on Aβ40 levels by ELISA among any of the co-cultures or the APP-SH-SY5Y cells alone (Figure 2.4C).

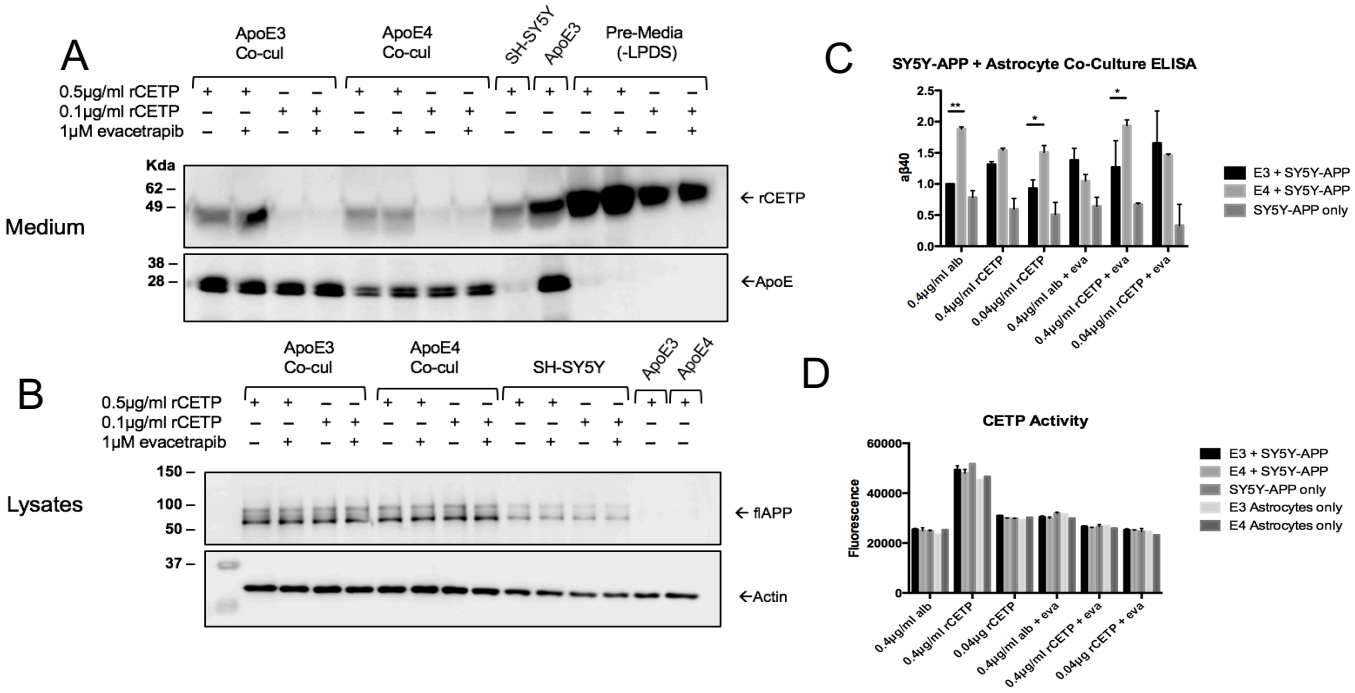


Figure 2.4: Murine hApoE astrocytes co-cultured with APP-SH-SY5Y and exogenous rCETP. (**A-B**) Western blot demonstrating medium and lysates 24 h after co-culturing murine hApoE astrocytes and APP-SH-SY5Y cells with rCETP with and without evacetrapib. Representative western blots of 3 biological replicates. (**C**) Aβ40 ELISA data from 24h co-cultures of APP-SH-SY5Y cells with hApoE murine astrocytes treated with BSA, rCETP, and/or evacetrapib (2-way ANOVA & tukey's multiple comparison mean ± SEM, n = 3). (**D**) CETP activity assay using conditioned media from co-cultures post-24h incubation (mean ± SEM, n = 3).

CETP Adeno-associated virus characterization

To investigate the potential role of peripheral CETP activity on AD pathology in mice, adeno-associated viruses were developed and characterized with a liver-specific promoter. The AAVDJ-hTGBprom-CETP(WT)-T2A-EGFP-WPRE virus, developed by the Neurophotonics Centre in Quebec (cloning and generation of virus), displayed moderate *in vitro* infectivity in HepG2 cells (Figure 2.5A). Indeed, as this viral plasmid included a T2A "self-cleaving peptide" element separating CETP and GFP, equal stoichiometric quantities of both GFP and CETP are produced under the liver-specific TGB promoter, and the green fluorescence of GFP can be clearly seen in HepG2 cells (Fig. 2.5A). However, secreted levels of CETP using this virus *in*

vitro were found to be quite low—CETP protein was only detectable by western blot after 12x concentration of serum-free media (Figure 2.5B). Furthermore, CETP activity was not seen from either the media (Figure 2.5C) or the concentrated media (data not shown) using the commercial activity assay. As well, no secreted CETP protein or activity was seen in the sera of transduced mice *in vivo* (Figure 2.6A, C), however both GFP and CETP protein were seen in the liver lysates of these mice (Figure 2.6B). Thus, we concluded that this viral construct is expressing the transgenes, but deficient in secreting CETP into the serum, which may be due to the T2A element within the plasmid (see discussion), we opted to develop our own set of viruses using an AAV CETP plasmid with liver-specific promoter that has been kindly provided by Dr. Rader and that was successfully used previously in literature (Rader AAV-TGB-CETP plasmid).

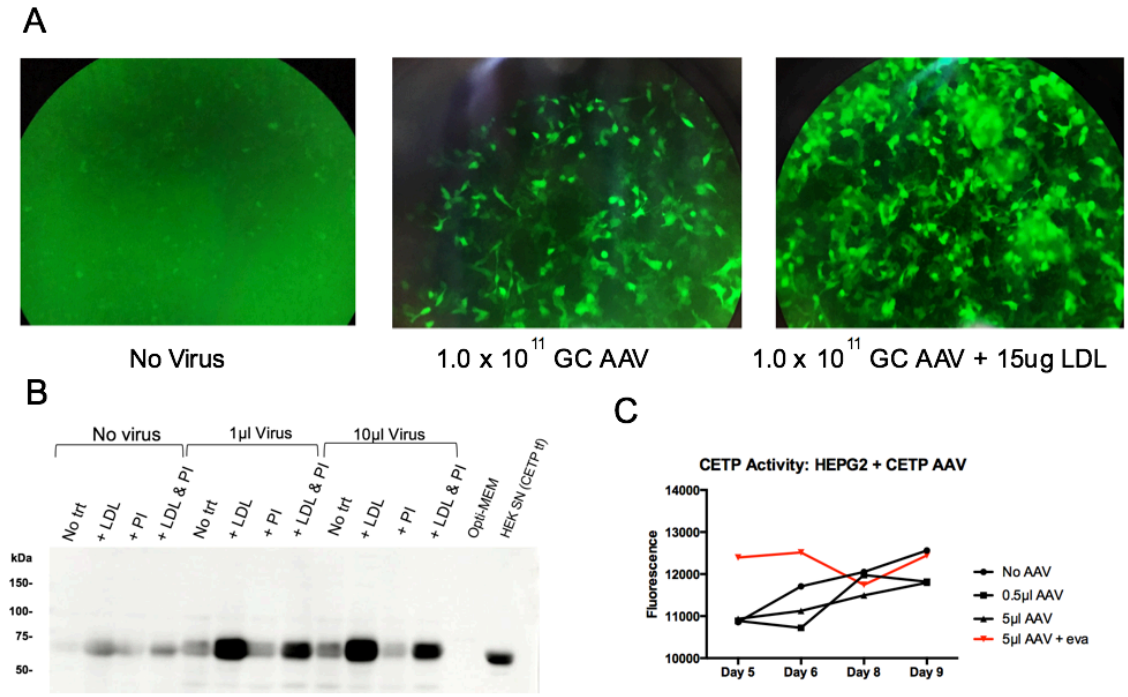


Figure 2.5: AAVDJ-hTGBprom-CETP(WT)-T2A-EGFP-WPRE virus is moderately infective *in vitro.* (**A**) Images of HepG2 through 10x ocular lens + 40x objective using a fluorescent microscope (~365/509nm) treated with and without LDL in serum-free media 8 days post infection using AAVDJ-TGB-GFP-CETP virus. (**B**) Western blot of 12x concentrated conditioned serum-free media of HepG2 cells treated with AAVDJ-TGB-GFP-CETP virus (titer: $1x10^{13}$ GC/ml). Cells were also treated with LDL and protease inhibitor to supplement HepG2 cells and to prevent CETP degradation. Medium collected 5 days post-transduction. (**C**) CETP activity in media from HepG2 cells post viral transduction with and without evacetrapib treatment.

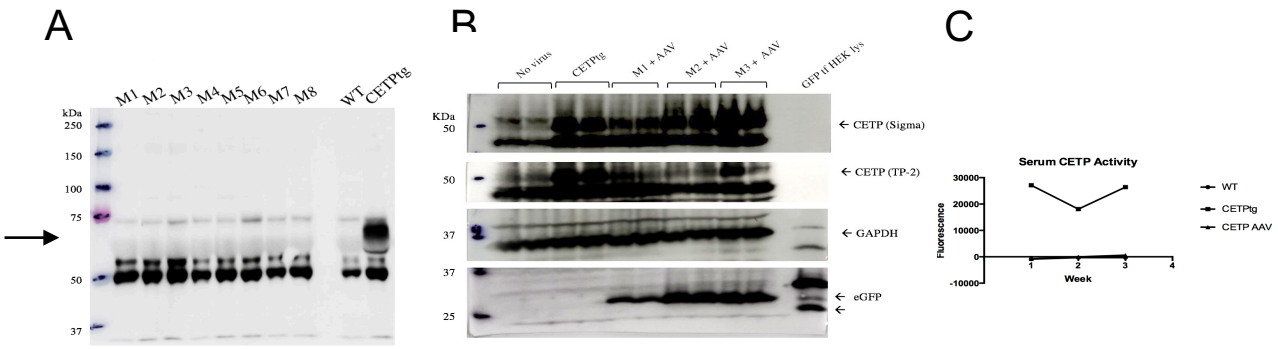


Figure 2.6: AAVDJ-TGB-GFP-CETP virus is not effective in vivo. (A) Western blot of 1:50 PBS-diluted mouse sera 9-weeks post injection of wild-type mice, with uninfected and CETP transgenic mice as negative and positive controls, respectively. Arrow indicates height at which CETP runs. (n=8) (B) Western blot of mouse liver lysates 9 weeks post injection demonstrating eGFP and CETP expression in transduced mice. (n=3) (C) CETP activity in mouse sera once every three weeks post viral transduction. (n=8)

A second set of CETP viruses were developed using the AAV plasmid developed by Daniel J. Rader which has been cited in previous publications (Khetarpal et al., 2016; Masson, Koseki, et al., 2009; Tanigawa et al., 2007). We mutated this plasmid using site-directed mutagenesis (Figure 2.7A) to develop three tunnel-mutants based on previously studied transfer activity modulations to CETP. When the ratio of WT CETP cholesteryl to triglyceride (CE/TG) transfer activity is set 1, the V198 CETP mutant has a CE/TG transfer ratio of 10 (1.0/0.1), the Q199A mutant has a CE/TG transfer ratio of 0.2 (0.25/1.1) and the T138Y mutant has a CE/TG transfer ratio of 0.9 (0.2/0.2). Thus, the V198W mutant is functionally "triglyceride transfer-deficient", the Q199A is "cholesterol-transfer deficient" and the T138Y is "inactive" (80% lower transfer activity) (Qiu et al., 2007) . These viruses would therefore be very useful in further characterizing which specific lipid transfer activity is responsible for any effects on Aβ seen. These mutated plasmids when transfected in HepG2 cells had cholesteryl linoleate transfer activity (i.e. cholesteryl ester transfer activity) consistent with the data reported by this group (Figure 2.7A, B). Post AAV-8 development using a AAV-2 Helper Free Packaging System, although the virus demonstrated very low *in vitro* transduction efficiency in HepG2 cells (data

not shown), *in vivo* transduction efficiency proved to be relatively high in mice—high levels of CETP activity were seen in injected wild-type mice as soon as one week post transduction (Figure 2.7C). From the 5 mutated viral plasmids, two viruses were purified to date by iodixonol density gradient ultracentrifugation (WT CETP AAV and GFP mock control) and the WT CETP AAV was characterized *in vivo*. 60μl of the CETP-WT virus at a calculated titer of approximately 10^{11}-10^{12} GCs/ml was injected in two WT mice (named AAV1 and AAV2) on a high cholesterol diet. 50 ul blood aliquots were taken every week and analyzed for CETP activity using a commercial CETP activity assay. Serum from a CETP tg mouse and from a WT (uninfected) mouse were also collected weekly as a positive and negative control, respectively. These mice were also kept on a high cholesterol diet. Expression of CETP through the virus was approximately 1.5-fold higher than then levels of a CETP transgenic mouse indicating the viral DNA construct as well as the production and purification of virus are effective.

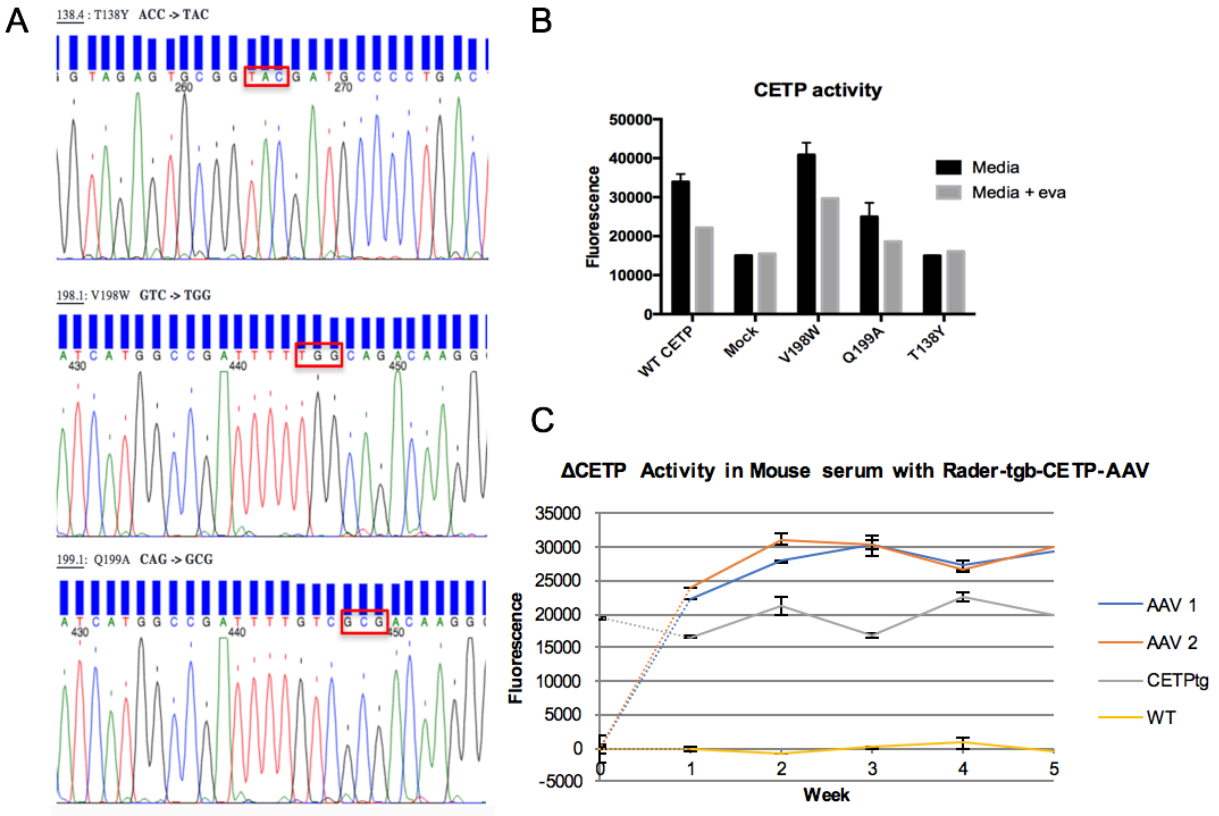


Figure 2.7: Rader AAV-TGB-CETP plasmid and mutants are effective *in vitro* and viral packaging is effective *in vivo*. (**A**) Sanger sequencing chromatograms of CETP gene within Rader AAV-TGB-CETP plasmid mutants after site-directed mutagenesis (**B**) CETP activity from 24 h conditioned HepG2 cells transfected with Rader AAV-TGB-CETP plasmid + mutants reflecting differences in tunnel mutant cholesteryl ester transfer abilities (mean ± SEM, n = 2). (**C**) CETP activity from mouse sera post transduction of wild-type Rader AAV-TGB-CETP plasmid with CETP tg and WT uninfected mice as controls. "AAV 1" and "AAV 2" denote the two mice injected with WT CETP AAV, "CETP tg" denotes serum collected from a CETP transgenic mouse (positive control) while "WT" denotes serum collected from a WT mouse not injected with virus. (mean ± SEM, n=2)

Discussion

The aim of this thesis was to identify a possible biochemical impact of extracellular CETP activity on astrocytic ApoE lipoproteins, and in turn on Aβ generation, in dependence of the ApoE isoform. Since the discovery of the ApoE allele being associated with increased risk for AD in 1993 (Strittmatter et al., 1993), the role of ApoE4 in disease pathology has remained enigmatic. However, there have been several studies evaluating the differences in ApoE-lipoprotein metabolism and cholesterol trafficking in an isoform-dependent manner. Previous genetic association studies have also reported interactive effects of CETP polymorphisms and ApoE genotype and risk for AD (Arias-Vásquez et al., 2007; E. A. Murphy et al., 2012). These studies are particularly noteworthy as the link between altered cholesterol and lipid metabolism and AD has been extensively corroborated, playing fundamental roles in both Aβ generation and tau phosphorylation (Gamba et al., 2012).

hApoE4 astrocytes produce more lipid droplets than hApoE3 astrocytes

LDs serve as storage reservoir for neutral lipids. In doing so, LDs buffer both cellular concentrations of fatty acids that would otherwise be lipotoxic if free in the cytoplasm and periods of starvation and stress wherein fatty acids are released for β-oxidation. These dynamic lipid-laden organelles have membrane contact sites with other organelles and are tightly coupled to cellular metabolism (Olzmann & Carvalho, 2019). Recent studies have found LDs appear to particularly accumulate along the brain-CSF interface of both human AD patients and 3xTg-AD mice compared to non-demented and WT mice respectively, which also appeared to correlate with impaired neural stem cell function at least for this AD mouse model (Hamilton et al., 2015). The link between LDs and ApoE isoform has not yet been widely explored, particularly in brain

cell types. For this thesis, I evaluated differences in LD size and levels in murine astrocytes expressing hApoE3 versus hApoE4.

Interestingly, we found that the hApoE4 astrocytes had many more and much larger LDs compared to the hApoE3 astrocytes both with and without LD stimulation using oleic acid, the quantification of which is currently ongoing. Similarly, other studies have found that using conditioned media from these hApoE astrocytes on wildtype human fibroblasts (which do not produce ApoE), hApoE4-conditioned media resulted in the formation of significantly increased LDs compared to hApoE3-conditioned media (Tambini et al., 2016). When cells uptake ApoE-containing lipoproteins through receptor-mediated endocytosis, hydrolysis occurs in peripheral endosomes and ApoE is degraded or recycled back to the cell membrane where it is re-lipidated by lipid transporters including ABCA1 and secreted (Heeren, Beisiegel, & Grewal, 2006). However, studies have shown that ApoE4 is recycled much less efficiently compared to ApoE3, resulting in intracellular lipid accumulation which may explain the increased number of LDs as a result of more available substrate (Heeren et al., 2003). This idea is supported by other studies using astrocytes with ABCA1 knocked-out which have been shown to have dramatically increased LDs compared to wild-type (Hirsch-Reinshagen et al., 2004).

Recently, another group established this increased LD phenotype seen in hApoE4 murine astrocytes versus hApoE3 and speculated that components of the lipid droplet monolayer may be a contributor to differences in LD size. As this group previously found that mice had significant differences in numerous lipid metabolism related pathways (including glycerophospholipid and sphingolipid metabolism) in an hApoE isoform-dependent fashion (L. A. Johnson, Torres, Impey, Stevens, & Raber, 2017), they theorized differences in *in vitro* LD phenotypes may be due to differences in both LD surfactant lipids including phosphatidylcholine which stabilize

LDs versus fusogenic lipids such as phosphatidic acid promote coalescence (Farmer et al., 2019). These differences may also be due to the differences in sources of energy between ApoE3 and ApoE4 astrocytes as a result of the differences in pathways; ApoE3 astrocytes may rely more heavily on fatty acid oxidation which would result in increased LD turnover. Furthermore, they hypothesized that as LDs have a similar structure to lipoproteins, perhaps ApoE plays a structural role in the integrity of LDs, and since ApoE4 astrocytes have been established as producing less ApoE protein which form smaller lipoproteins than those of ApoE3, the lower intracellular quantity of ApoE in ApoE4 astrocytes may modulate LD count and size (Farmer et al., 2019).

Cell-free assessment of CETP-mediated modulations of Astrocytic Lipoproteins

To see if CETP is capable of exerting an extracellular effect on endogenously secreted astrocytic lipoprotein particles, cell-free assays with recombinant CETP were set up to control for confounding variables that may arise from a CETP transfection or stable cells. Interestingly, it was seen that in the 280 nm chromatograms, there was a modulation in the lipoprotein peaks different from the modulation seen in the ApoE4 media with CETP activity inhibition. Indeed, for both the ApoE3 and ApoE4 conditioned media, presence of CETP appeared to coalesce two discrete lipoprotein groups creating intermediate particles as evidenced by the eliminated concavity seen between the spectrum lipoprotein peaks in the "E3/E4 alone" baseline spectra. However, the spectral "rescue" using CETP inhibitors ameliorated this effect only with the E3 lipoprotein particles and not with the E4. It is possible for this to have been due to differences in affinity for CETP to ApoE3 versus ApoE4 particles compared to CETP's affinity to evacetrapib, perhaps due isoform-dependent differences in lipoprotein structure or lipid composition. As such, evacetrapib is effective in displacing CETP for ApoE3 particles, but it is ineffective in

removing CETP from ApoE3 particles. This could be evaluated using binding kinetic analysis techniques such as Surface Plasmon Resonance (SPR) where rCETP would be set up as the conjugated ligand and recombinant ApoE3 and ApoE4 in solution or ApoE-conditioned media is used as the analyte. Interestingly, in some biological replicates of this experiment, the 280 nm peak seen at an elution volume of 8 ml appeared to also be amplified by the presence of CETP in E3 samples but not E4 samples. This peak was seen to be approximately 1.2x larger in both "E3 + rCETP" and "E3 + rCETP + eva" spectra compared to "E3", implying it is a VLDL-sized lipoprotein particle also expressed by these cultured astrocytes. In David Holtzman's original characterization of these astrocytes, these peaks were also seen in both the phospholipid and total cholesterol quantification from the size-exclusion fractionation, though as these fractions did not seem to contain apolipoproteins, they were discounted as "large cell membrane fragments" rather than large lipoproteins (Fagan et al., 1999). However, other studies using human astrocytes have found lipoproteins in the range of plasma VLDL as determined by gel filtration chromatography, transmission electron microscopy, gradient gel electrophoresis, and cholesterol quantification assays (Shachter et al., 2004).

Co-cultures using CETP have no effect on Aβ levels

In contrast to what was seen in our preliminary *in vivo* data using CETP/APP double transgenic mice wherein Aβ levels were significantly increased, the *in vitro* co-culture data presented here did not result in CETP activity-dependent increases in Aβ40 levels. As CETP lipid exchange is thought to be a passive process dependent on the relative levels of triglycerides and cholesteryl esters in respective lipoproteins, it is possible that the lipoproteins secreted by these hApoE lipoproteins were not reflective of the entire lipoprotein pool found in the *in vivo* brain. Indeed, while these particular murine astrocytes have been characterized as secreting

distinct classes of ApoE and ApoJ-containing lipoproteins(Fagan et al., 1999), microglia are also known to produce relatively high amounts of ApoE-containing lipoproteins and even "LDL-like particles" (Ulrich et al., 2018; Q. Xu, Li, Cyras, Sanan, & Cordell, 2000; Ye Zhang et al., 2014). Furthermore, there are several lines of evidence suggesting ApoA-I is capable of crossing the blood-brain barrier from the periphery into the CNS as this apolipoprotein has consistently been found in mammalian CSF whereas ApoA-I mRNA has not been found in the brain (Pitas, Boyles, Lee, Hui, & Weisgraber, 1987; Stukas et al., 2014). As such, it is possible that the isolated *in vitro* co-culture systems presented here do not accurately represent the heterogeneity of lipoprotein substrates for CETP, an enzyme whose activity is contingent on diverse lipoprotein classes and their respectively diverse lipidation. Thus, CETP's action within this closed *in vitro* system may not yield any net lipid transfer for these HDL-like particles that could possibly exert an effect on the APP-SH-SY5Ys. Dr. Oestereich's preliminary *in vitro* data with Human embryonic kidney 293 cells (HEK294T) cells supports this idea as well: when these cells were co-transfected with hAPP and CETP, a significant increase in the generation of Aβ38, Aβ40 and Aβ42 was seen when FBS was present in the cell culture supernatant. This effect was also reversible through the addition of CETP inhibitor (evacetrapib) or using CETP transfer-deficient mutants. However, when FBS was replaced with LPDS, the CETP-mediated increase in Aβ levels was lost (Oestereich, unpublished). In the case of HEK293T cells, an immortalized derivative of human kidney cells, this can be easily rationalized as peripheral cells (with the exception of liver cells) uptake LDL particles through the LDL receptor pathway—they generally do not uptake HDL (Lestavel & Fruchart, 1994). Therefore, when FBS is in the media, CETP activity can confer a modulation on bovine LDL (low in cholesterol) lipid levels with the presence of bovine HDL (high in cholesterol) because these lipoprotein species are so

contrasting in terms of lipidation. As a result, the LDL post-CETP activity that is uptaken by HEK cells is enriched with cholesterol, and this increase in intracellular cholesterol confers downstream effect on Aβ production. It should be noted that this effect necessarily requires two requisites: 1) Lipoprotein classes that are relatively different in terms of lipidation such that CETP activity can confer a net effect; 2) The Aβ-producing cell type in question must exclusively (or at least preferentially) uptake only one of these lipoprotein classes. With LPDS, CETP cannot perform any net transfer with no lipoprotein substrates available. In the context of the murine lipoproteome and specifically for that of the CNS, things are much less clear. As the peripheral cells of mice uptake lipids from HDL as opposed to LDL as seen in humans, whether the apolipoprotein receptors of peripheral or neuronal cells parallel the lipoprotein class specificity seen in human peripheral and neuronal cells requires further research. Furthermore, whether the CNS lipoproteome is diverse enough for CETP to be able to exert a net effect is also unclear. Levels of CETP in the brain must also be high enough to exert an effect, in addition. It is also possible that the effect seen with the CETP/APP double transgenic animals was due to peripheral actions that have downstream effects in the brain. For example, increases in peripheral murine LDL levels may have resulted in increases in peripheral inflammation around or at the blood-brain barrier which may have produced cytokines that in turn caused neuro-inflammation and increases in Aβ levels. This will be confirmed through trials using the CETP AAV + mutants characterized in this thesis. Finally, it is possible that the ApoE lipoproteins produced by these mouse astrocytes are overly artificial due to its chimeric properties (i.e. human apolipoprotein E whilst all other associated proteins are murine) resulting in CETP's effect being lost. Indeed, in Dr. Holtzman's original characterization of these lipoproteins, it was found that these astrocytes contain less lipids than those derived from wild-type mice with murine ApoE in spite of there

being more ApoE secreted from the E3 and E4 astrocytes (Fagan et al., 1999). Thus, mouse ApoE supports the production of lipoprotein particles with a greater lipid:ApoE profile than human ApoE in a mouse cell context. It should be noted that out of 299 amino acids in ApoE, mouse ApoE shares only a 70% sequence homology with human ApoE4 (Liao et al., 2015)

CETP AAV characterization and development

To discern if the increases in Aβ levels in brains of double transgenic hAPP/CETP mice relative to Aβ levels in the brains of hAPP mice is due to CETP activity in the brain or CETP activity in the periphery, where protein levels are much higher, AAV's with liver-specific promoters were characterized to evaluate the effects of exclusively systemic expression of CETP. The AAVDJ-hTGBprom-CETP(WT)-T2A-EGFP-WPRE virus was found to have relatively high *in vitro* transduction efficiency, however *in vivo* no CETP activity or secreted protein could be detected, despite GFP and CETP protein being seen in liver lysates. This could possibly be due to the T2A "self-cleaving" peptide that separates GFP and CETP such that equimolar amounts of both proteins are produced from a single transcript. This peptide is thought to function by causing the ribosome to 'skip' synthesis of the final glycyl-prolyl peptide bond of the 21-amino acid sequence, causing the protein upstream of this element to have an additional 20-amino acid T2A sequence attached to its C-terminus while the protein downstream has an additional proline at its N-terminus. Although these sequences are generally supposed to have minimal functional consequences, there are several studies that have shown problems can arise when using polycistronic linkers including T2A to separate proteins with differing targeted cellular localizations (de Felipe, Luke, Brown, & Ryan, 2010; de Felipe & Ryan, 2004; Rothwell et al., 2010). As well, one of these studies found that the order of these transgenes could also impact secretion and functionality; when the transforming growth factor beta (TGF-β) cytokine

was placed upstream GFP separated by the T2A element, secretion and functionality was found to be dramatically reduced. However, when GFP was upstream of TGF-β and separated with this T2A linker, protein levels for this cytokine were normal. In both cases, GFP did not seem to affected by its relative position in both vectors (Rothwell et al., 2010). It is possible that the additional T2A sequence added to CETP may impair its folding or post-translational modifications necessary for secretion. Contrastingly, the Rader AAV-TGB-CETP plasmid demonstrated high degrees of CETP secreted protein expression *in viv*o but little to no CETP from *in vitro* transduction was seen in HepG2 cells. This plasmid does not contain a fluorescent protein or a T2A element. Interestingly, although both plasmids had identical TGB promoter sequences, the CETP transcript sequences were slightly different in that the Rader plasmid sequence includes additional flanking sequences of approximately 60 nucleotides which may have played a role in proper protein maturation. The differences in *in vitro* versus *in vivo* transduction efficiency can be explained by the differences in viral serotypes. Whilst the AAVDJ-hTGBprom-CETP(WT)-T2A-EGFP-WPRE virus was of AAV-DJ serotype, the Rader plasmid was of AAV-8 serotype. In HepG2 cells, the AAV-DJ serotype has been reported to have over 4000x *in vitro* infectivity relative to the AAV-8 serotype. However, both serotypes are expected to have relatively high tropism in liver *in vivo* (Grimm et al., 2008).

Future Outlook

In light of the lipid droplet phenotype being seen with the hApoE murine astrocytes wherein hApoE4 astrocytes produce larger and more lipid droplets than hApoE3 astrocytes, further experiments should be conducted to expand on our knowledge on how ApoE plays a role in AD. Indeed, comparisons of lipid-related gene expression levels as well as cellular metabolism would provide valuable insight as to why or how these astrocytes vary in terms of lipid storage so substantially. This could be done through qPCR for lipid-related genes as well as lipidomics. Furthermore, it would be interesting to see if CETP activity plays a role in lipid droplet formation or modulation. This could be done through both addition of exogenous rCETP or CETP transfections. Ideally, to see if there is an ApoE isoform-dependent effect on Aβ generation, it would also be extremely elucidating to cross CETP tg mice with hApoE3 and hApoE4 mice. These mice could have their CSF collected and CNS lipoprotein profiles compared in terms of lipidation and morphology. To go a step further, triple transgenic mice for CETP, hApoE and hAPP could also be generated to see relative effects on Aβ generation.

Another direction to take this project would be through the use of the characterized CETP AAVs. Indeed, within this master's thesis, 5 AAVs were developed: WT CETP, inactive CETP, cholesterol transfer-deficient CETP, triglyceride transfer-deficient CETP, and GFP (negative control). These viruses should be used in a trial with APP-transgenic mice to investigate whether peripheral CETP activity plays a role in Aβ levels in the brain. If indeed it is the low amounts of CETP in the brain that confers the 5-fold increase in Aβ levels *in vivo*, then using these liver-specific viruses should not impact brain Aβ levels. However, if peripheral CETP activity is the cause for the Aβ elevation, then the effect should be almost entirely abrogated using the CETP inactive mutant. In this case, the cholesterol and triglyceride transfer-deficient viruses would

provide further insight as to which class of lipid transfer plays the predominant role in Aβ modulation. Finally, if no effect is seen with peripheral CETP activity on brain Aβ levels, it may be worthwhile to develop CETP viruses with astrocyte-specific promoters to isolate CETP activity to the CNS. These models of segregated CETP activity in the CNS versus periphery through the use of AAVs is vital for the possibility of pharmacological intervention in terms of CETP inhibition.

Conclusion

In our lab, it was found that CETP x hAPP double transgenic mice have increased levels of brain Aβ compared to hAPP mice. The goal of this master's thesis was to discern if there is an interaction between CETP and Aβ generation in dependence of ApoE status. This was done using both *in vitro* co-culture models as well as through the development of adeno-associated CETP viruses to restrict CETP expression to the periphery of mice. I hypothesized that the change in Aβ levels was due to the low levels of CETP in the brains rather than the higher CETP levels in the blood of these mice. However, although subtle modulations in lipoproteins were seen, *in vitro* the presence of active rCETP did not impact Aβ40 levels when SH-SY5Ys were cultured with hApoE-expressing astrocytes of either E3 or E4 isoform. This may either be due to the fact that this *in vitro* system did not include all of the cell types or components required to reproduce the effect seen in the brains of these mice with CETP, or because the effect of CETP on mice is indeed peripheral instead. To test this, a WT CETP virus and CETP mutant viruses were developed and characterized for use in a subsequent trial to test whether blood CETP alone is capable of modulating Aβ40 levels.

Although whether or not CETP may have a potent role in Alzheimer's disease is unclear, the CETP transgenic mouse exists as a much better model for AD, a disease so connected to lipid metabolism. The research done in this thesis goes towards the advocacy for AD research with model systems that more comprehensively simulate the metabolic profile of demented patients.

Abbreviations

ACT	Actin-Like Protein
AAV	Adeno-Associated Virus
AMP	Adenosine Monophosphate
AMPK	Adenosine Monophosphate-Activated Protein Kinase
ATP	Adenosine Triphosphate
AD	Alzheimer's Disease
APP	Amyloid Precursor Protein
ApoA-I	Apolipoprotein A-I
ApoB	Apolipoprotein B
ApoC-II	Apolipoprotein C-II
ApoE	Apolipoprotein E
ABCA1	ATP-Binding Cassette Transporter
BACE1	B-Site APP-Cleaving Enzyme 1
BODIPY	Boron-Dipyrromethene
BSA	Bovine Serum Albumin
CAIDE	Cardiovascular Risk Factors, Aging, And Incidence of Dementia
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CETP	Cholesteryl Ester Transfer Protein
DMEM	Dulbecco’s Modified Eagle Medium
EOAD	Early Onset Autosomal Dominant Familial Alzheimer's Disease
ER	Endoplasmic Reticulum

ELISA	Enzyme-Linked Immunosorbent Assay
EDTA	Ethylenediaminetetraacetic Acid
FPLC	Fast Protein Liquid Chromatography
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GFP	Green Fluorescent Protein
HL	Hepatic Lipase
HDL	High Density Lipoproteins
HRP	Horseradish Peroxidase
HMG-CoA	Hydroxy B-Methylglutaryl-Coenzyme A
DAG	Diacylglycerol
IPSC	Induced Pluripotent Stem Cell
IP_3	Inositol Triphosphate
INSIG	Insulin Induced Gene 1
IDL	Intermediate-Density Lipoproteins
ITR	Inverted Terminal Repeat
LOAD	Late Onset Sporadic Alzheimer's Disease
LRP	LDL Receptor-Related Protein
LCAT	Lecithin–Cholesterol Acyltransferase
LPDS	Lipoprotein Deficient Serum
LPL	Lipoprotein Lipase
LDLR	Low-Density Lipoprotein (LDL) Receptor
LDL	Low-Density Lipoproteins
LB	Lysogeny Broth
MTTP	Microsomal Triglyceride Transfer Protein

PBS	Phosphate-Buffered Saline
PLC	Phospholipase C
PEI	Polyethylenimine
PCR	Polymerase Chain Reaction
PKC	Protein Kinase C
SR-B1	Scavenger Receptor Class B Type 1
SEQ	Sequencing Primers
SNP	Single-Nucleotide Polymorphism
S1P	Site-1 Protease
S2P	Site-2 Protease
SDM	Site-Directed Mutagenesis
sAPP-α	Soluble APP-A
SCAP	SREBP Cleavage-Activating Protein
SRE	Sterol Regulatory Element
SREBP	Sterol Regulatory Element Binding Protein
TGB	Thyroglobulin Binding Globulin
TGF-β	Transforming Growth Factor Beta
TMB	3,3',5,5'-Tetramethylbenzidine
VLDL	Very-Low-Density Lipoproteins
WT	Wild-Type
AMPA	A-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid

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