The Role of High-Density Lipoproteins and

Related Pathways in Alzheimer's Disease

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Dedication

This dissertation is dedicated to my wife, Astia, whose continued support and encouragement has been the bedrock of my graduate education.

Abstract

Alzheimer's disease (AD) is the most prevalent age-related dementia and will place an increasingly demanding burden on our healthcare system as the population ages. It has been firmly established that high plasma levels of high-density lipoprotein (HDL) protect against cardiovascular disease and accumulating evidence indicates that the beneficial role of HDL extends to the central nervous system. There are several important biological mechanisms that regulate HDL generation and metabolism/function. One is through the cholesteryl ester transfer protein (CETP), which transports cholesterol esters and triglycerides between different lipoprotein particles. Loss-of-function mutations in CETP are associated with better cognition in aging. To investigate the role of CETP in AD, human CETP transgenic mice were crossed with an Alzheimer's mouse model, followed by biochemical and behavioral analyses. The results showed that CETP-induced modest decrease in plasma HDL levels was insufficient to affect brain amyloid pathology, neuroinflammation, or memory function. Next, to explore the therapeutic potential of a cardiovascular protective, HDL-mimetic-peptide called D-apoJ[113-122], AD mice were treated with the peptide. This treatment robustly reduced brain amyloid pathology and improved memory function in AD mice. Further analyses showed that D-apoJ[113-122] exerted its beneficial effects through reduction of cerebral vascular amyloid deposition and clearance of brain amyloid to plasma. Finally, prenyltransferase-deficient mice were used to investigate the role of protein prenylation in synaptic function. Prenylation is an important posttranslational lipid modification process that attaches isoprenoids (the intermediates in the cholesterol biosynthesis pathway) to target proteins.

Electrophysiological/histochemical experiments showed that systemic or forebrain-specific deficiency of one particular prenyltransferase, geranylgeranyltransferase-1, caused marked impairment in hippocampal synaptic plasticity and decrease in neuronal dendritic spine density. Further analyses indicated that reduction of prenylation of certain small GTPases, which rely on prenylation for proper cellular localization and function, underlies the detrimental effects in these mice, as observed in aged mouse brains. These results corroborate the critical role of protein prenylation in synaptic function during development and in the adult brain. Taken together, findings from this research provide novel insights into the role of HDL and related pathways in the pathogenesis of AD, and offer new avenues to develop effective therapies for AD.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Aβ, Amyloid-β peptide

ABC, ATP-binding cassette

ABCA1, ATP-binding cassette type A1

ABCB1, ATP-binding cassette type B1

ABCG2, ATP-binding cassette type G2

AD, Alzheimer's disease

AICD, Amyloid precursor protein intracellular domain

AKT, Protein kinase B

ALS, Amyotrophic lateral sclerosis

AMPA, Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

RMANOVA, Repeated measures analysis of variance

AO, Apical oblique

apo, Apolipoprotein

apoA-I, Apolipoprotein A-1

apoE2/3/4, Apolipoprotein E2/3/4

apoJ, Apolipoprotein J

APP, Amyloid precursor protein

α2M, Alpha 2-macroglobulin

β-CTF, Beta-secretase cleavage product c-terminal fragment

BACE1, Beta-secretase 1

BBB, Blood brain barrier

BCRP, Breast cancer resistance protein

BDNF, Brain-derived neurotropic factor

BS, Basal shaft

CAA, Cerebral amyloid angiopathy

CAD, Coronary artery disease

CaMKII, Ca2+/calmodulin-dependent protein kinase

CE, Cholesterol esters

CETP, Cholesteryl ester transfer protein

ChE, Cholinesterase

CLU, Clusterin or apoJ

CNS, Central nervous system

CSF, Cerebrospinal fluid

cyp, Cytochromes P450

E-LTP, Early long-term potentiation

ERK, Extracellular signal-regulated kinase

FC, Unesterified free cholesterol

fEPSPs, Field excitatory post-synaptic potentials

FPP, Farnesyl pyrophosphate

FT, Farnesyl transferase

FTI, Farnesyl transferase inhibitors

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase

GAPs, GTPase-activating proteins

GDP, Guanosine diphosphate

GEFs, Guanine nucleotide exchange factors

GFAP, Glial fibrillary acidic protein

GGPP, Geranylgeranyl pyrophosphate

GGPPS, Geranylgeranyl pyrophosphate synthase

GGT-1, Geranylgeranyl transferase-1

GGT-2, Geranylgeranyl transferase-2 or RabGGT

GGTI, Geranylgeranyl transferase-1 inhibitors

Glu, Glutamate

GTP, Guanosine triphosphate

GWAS, Genome-wide association studies

HDL, High-density lipoprotein

HDL-C, High-density lipoprotein cholesterol

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

I/O, Input/output

IACUC, Institutional animal care and use committee

IDE, Insulin degrading enzyme

IVUS, Intravascular ultrasound

LCAT, Lecithin cholesterol acyltransferase

LC-MS, Liquid chromatography and mass spectrometry

LDL, Low density lipoprotein

L-LTP, Late long-term potentiation

LRP1, Low density lipoprotein receptor-related protein 1

LTD, Long-term depression

LTP, Long-term potentiation

LXRß, Liver X receptor beta

LXRα, Liver X receptor alpha

MAPK, Mitogen-activated protein kinases

MCI, Mild cognitive impairment

miRNAs, MicroRNAs

Mo, Months old

NEP, Neprilysin

NMDA, N-methyl D-aspartate

PD, Parkinson's disease

PDGF-β, Platelet-derived growth factor β

PGP, P-glycoprotein

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

PKC, Protein kinase C

PL, Phospholipids

PLTP, Phospholipid transfer protein

PON1, Paraoxonase 1

PPARα, Peroxisome proliferator-activated receptor alpha

PPF, Paired pulse facilitation

PS1, Presenilin-1

PS2, Presenilin-2

P-tau, Phosphorylated tau

RAGE, Receptor for advanced glycation endproducts

RCT, Reverse cholesterol transport

rHDL, Recombinant high-density lipoprotein

ROCK, RhoA effector rho-kinase

RXR, Retinoid X receptors

sLRP1, Soluble low-density lipoprotein receptor-related protein 1

SNP, Single nucleotide polymorphism

SR-B1, Scavenge receptor B1

SS, Squalene synthase

TBS, Theta burst stimulation

TG, Triglycerides

TrkB, Kinase receptor B

TTR, Transthyretin

WT, Wild type

CHAPTER 1 – LITERATURE REVIEW

Overview of Alzheimer's disease:

Alzheimer's disease (AD), the most common neurodegenerative disease (Alzheimer's Association 2017), is often associated with impaired memory and other neurological deficits. The prevalence of AD is increasing as the global population ages. AD was first described in 1906, when Dr. Alois Alzheimer described unusual pathological features that were later described as amyloid plaques and neurofibrillary tangles. These became widely accepted as the major pathological hallmarks of AD. Clinically, AD is characterized by progressive cognitive impairment, and is confirmed by the presence of plaques and tangles in autopsied brain.

A particular focus has been devoted to the development and clearance of amyloid plaques that are comprised of small amyloid β (Aβ) peptides that aggregate into insoluble plaques that damage the surrounding tissue. There has been intense study into Aβ formation, aggregation, and clearance as key points for disease intervention. This line of thinking resulted from the amyloid cascade hypothesis that emphasizes the role of Aβ in AD pathogenesis (Hardy and Higgins 1992; Hardy and Selkoe 2002). Aβ is derived from the abnormal processing of the amyloid precursor protein (APP) by membrane secretase activity (Kang et al., 1987). The two primary secretases in the amyloidogenic pathway include β-secretase 1 (BACE1) and γ-secretase. γ-Secretase consists of at least four subunits: presenilin, nicastrin, anterior pharynx-defective 1, and presenilin enhancer 2 (Kaether, Haass, and Steiner 2006). The Aβ fragments formed from β- and γ- secretase

activity on APP are highly toxic, resulting in neuronal cell death (O'Brien and Wong 2011). However, memory impairments associated with AD begin prior to the accumulation of plaques and cell death (Haass and Selkoe 2007; Ashe and Zahs 2010). Additional evidence shows that the level of extracellular soluble Aβ fragments directly correlate with cognitive decline (Selkoe et al., 2002), and hypothesize that soluble Aβ is responsible for the onset of AD symptoms.

AD symptoms begin with the dysfunction of cholinergic and glutamatergic synapses in the hippocampus and neocortex before neuronal degeneration (Selkoe et al., 2002). Recent evidence reports that this synaptic dysfunction is caused by soluble oligomeric Aβ (Haass and Selkoe 2007; Ashe and Zahs 2010). It has been shown that memory and cognitive deficits correlate better with soluble cortical Aβ concentration (Selkoe et al., 2002) and tau tangles load (Nelson et al., 2013) than senile (insoluble) plaques. Electrophysiological studies in young APP transgenic (tg) mice have revealed significant hippocampal synaptic deficits well before the development of microscopically detectable Aβ deposits (Jacobsen et al., 2006; Hsai et al., 1999; Chapman et al., 1999). However, the cellular and molecular mechanisms underlying synaptic dysfunction caused by Aβ oligomers and tau tangles are not fully understood. Additional pathological findings in AD will be described later in this chapter.

Familial vs sporadic Alzheimer's disease:

There are two forms of AD which are commonly referred to as familial and sporadic (Mayeux and Stern 2012). Familial AD is caused by heritable mutations in one of several

well characterized genes responsible for increased amyloid accumulation such as the APP, presenilin-1 (PS1) and presenilin-2 (PS2) (Waring and Rosenberg 2008) and is also referred to as early onset AD. Early onset AD symptoms begin before age 65. The average age for early onset symptoms to appear is around 55 years of age (Koedam et al., 2010). Typically, patients with familial forms of AD have a higher Aβ42/40 ratio, indicating a larger accumulation of the more toxic Aβ42 species relative to total amyloid burden. In a late onset AD patient, symptoms appear after age 65 (average age 75) with Aβ40 as the most prevalent species of the peptide (Koedam et al., 2010). Overall, mutations that lead to familial AD are relatively rare in the general population and, therefore, represent approximately 1-5% of the total AD patient population (Alzheimer's Association 2017).

Age is the biggest risk factor for developing AD (Alzheimer's Association 2017), with half of all people over 85 developing the disease (Hebert et al., 2003). One of the major genetic risk factors in late-onset AD is the apolipoprotein E4 (apoE4) isoform. A single copy increases one's risk of AD by three times, and two copies of apoE4 increases the overall risk by 15 times (Saunders et al., 1993). The apoE4 allele is found in about 15% of the general population, but that frequency is up to 50% in AD patients, indicating a strong link between apoE genotype and AD risk (Saunders et al., 1993). ApoE4 is also a major risk factor for many negative cardiovascular outcomes, indicating there may be a substantial biological link between these two fields (Mahley and Rall 2000).

Pathological hallmarks of Alzheimer's disease:

A small amount of Aβ production is normal in all mammalian brains (Haass and Selkoe 1993), which is derived from secretase cleavage of the parent protein, APP (Thinakaran and Koo 2008; Haass et al., 2012). Aβ peptides aggregate in the brains of pre-AD patients to form senile plaques that are spherical extracellular lesions. These diffuse senile plaques are one of the earliest pathological markers and precede cognitive impairment (Jack et al., 2010; Mann et al., 1988). Aβ continues to accumulate with aging primarily in the posterior cingulate, followed by the lateral temporal lobe, and finally the frontal lobe. (Jack et al., 2010).

Interestingly, plaque pathology is not unique to AD (Selkoe et al., 1987) and, therefore, Aβ plaques alone are not sufficient to diagnose a patient with AD. Co-lesions with neurofibrillary tangles, age of symptom onset, and cognitive assessments are required for a typical AD diagnosis. Aβ production is increased in response to oxidative stress (Castellani et al., 2006) and Aβ's clearance is slowed with age (Patterson et al., 2015) and in patients with AD (Selkoe 2001; Tanzi et al., 2004; Mawuenyega et al., 2010). Several key enzymes are responsible for the degradation of Aβ, such as neprilysin (NEP) (Iwata et al., 2000, 2001) and insulin degrading enzyme (IDE) (Farris et al., 2003). The net Aβ levels in the brain are a result of the balance between production and clearance and several novel drug development projects have aimed at altering that balance to favor decreasing the Aβ burden in AD brain (Orgogozo et al., 2003).

Under normal conditions, tau is a microtubule-associated protein that is important for stabilizing neuronal axons and the cell's cytoskeletal features. In AD, misfolded/hyperphosphorylated tau forms the core of intracellular neurofibrillary tangles (Grundke-Iqbal et al., 1986; Wood et al., 1986). Biochemical changes in tau levels and phosphorylation occur after Aβ levels increase, but prior to initial synapse and neuron loss (Gómez-Isla et al., 1997; Iqbal and Grundke-Iqbal 2002) although causative factors have not been fully elucidated (de Calignon et al., 2009). High cerebrospinal fluid (CSF) levels of tau have been associated with a quicker disease progression from mild cognitive impairment (MCI) to AD (Blom et al., 2009). Furthermore, in AD patients, high tau levels are associated with faster cognitive decline and higher overall mortality (Sämgård et al., 2010; Wallin et al., 2009). Similar disease progression patterns are seen in patients with higher levels of phosphorylated tau (p-tau) (Sämgård et al., 2010; Blom et al., 2009). Taken together, there is a need to better understand AD development in order to find the critical tipping points where treatments can still be effective.

Aβ peptide is capable of forming deposits outside of the brain parenchyma. One major site of such deposits is the vessel walls of brain capillaries in a condition known as cerebral amyloid angiopathy (CAA). These vascular deposits consist primarily of Aβ40 and accumulate in approximately 80% of AD patients (Serrano-Pozo et al., 2011). Cortical capillaries and small arterioles are most commonly affected by CAA and result in diminished vascular efficiency (Olichney et al., 2000). There have been several studies that found a positive correlation between cognitive decline in AD with post-mortem CAA levels (Pfeifer et al., 2002; Arvanitakis et al., 2011; Greenberg et al., 2004).

Aβ and tau mediated neuronal injury occurs before cognitive and structural damage (Jack et al., 2010). Localized neuronal and microglial injury are associated with early senile lesions (Probst et al., 1987). Such injuries 'activate' microglia cells that are found at the core of early plaques (Eikelenboom et al., 2010). Accumulating information suggests that inflammation plays a key role in early AD pathogenesis (Heneka et al., 2015) and could be an important mechanism underlying AD symptoms.

Amyloid precursor protein trafficking and processing:

APP trafficking and processing are modulated by a number of mechanisms (Small and Gandy 2006; Haass et al., 2012; Cam and Bu 2006). One of the mechanisms is cell membrane fluidity, regulated mainly by the cholesterol content. While the non-amyloidogenic cleavage of APP by α-secretase occurs in cholesterol-poor and phospholipid-rich domains, the amyloidogenic cleavages by β- and γ-secretases are preferred in the cholesterol-rich domains (lipid rafts) (Wolozin 2001). Another controlling mechanism for APP processing is the distinct localization of secretases. α-Secretase activity is located primarily at the cell surface, whereas β- and γ-secretase activities are found mainly in membranous compartments (e.g., endosomes) inside the cell (Cam and Bu 2006; Haass et al., 2012; Small and Gandy 2006). Modulation of APP processing has for many years been a main biochemical target for the development of drugs for the treatment of AD. The goal is to favor the production of non-amyloidogenic fragments resulting from cleavage of APP by α-secretase over amyloidogenic Aβ that is produced by β-secretase (Chow et al., 2010).

FDA-approved treatments to delay symptoms of Alzheimer's disease:

Over the last three decades, several drugs have been approved by the FDA for the symptomatic treatment of AD. Importantly, none of these drugs are capable of delaying or preventing AD progression. Currently approved drugs fall into two categories, namely acetylcholinesterase inhibitors or NMDAR antagonists (**Table 1.1**). Donepezil, galantamine, rivastigmine, and tacrine are all acetylcholinesterase inhibitors that work to preserve the limited amount of the neurotransmitter acetylcholine in the brains of AD patients (Raskind et al., 2000; Burns et al., 1999; Camps and Muñoz-Torrero 2002). Acetylcholine is reduced as the neurons releasing the neurotransmitters slowly die due to Aβ-induced toxicity (Whitehouse et al., 1981, 1982; Wong et al., 1999). Inhibiting acetylcholine breakdown works to temporarily 'boost' the signal that is released from the remaining neurons. However, as more neurons continue to die while a patient's AD progresses, these drugs becomes less and less effective at masking symptoms (Sun et al., 2008). Memantine is an NMDA receptor antagonist that blocks the excitotoxicity that is seen in moderate to severe AD (Rogawski and Wenk 2003). Blocking excitotoxicity helps to prevent the emergence of hallucinations and other behavioral symptoms of AD, although the efficacy is lower than originally thought (Gauthier et al., 2008; Winblad et al., 2007).

Table 1.1 - List of currently approved Alzheimer's disease drugs:

Name	Year Approved	Targeted System	Conditions	Stage of AD
Donepezil	1996	Acetylcholinesterase inhibitor	Alzheimer's disease	Mild, moderate, severe
Galantamine	2004	Acetylcholinesterase inhibitor	Alzheimer's disease	Mild to moderate
Rivastigmine	2000	Acetylcholinesterase inhibitor	Alzheimer's disease Parkinson's disease dementia	Mild to moderate
Tacrine	1994 -2013 (discontinued)	Acetylcholinesterase inhibitor	Alzheimer's disease	NA
Memantine	2003	NMDAR antagonist	Alzheimer's disease	Moderate to severe

Transgenic mouse models of Alzheimer's disease:

Transgenic animal models of any disease state attempt to recreate key pathological hallmarks of the human-form of the disease. In transgenic mouse models, this is typically achieved via overexpression of inserted genetic mutations observed in familial cases of AD. This allows researchers to identify important disease mechanisms and define targets for drug development against the human disease. AD transgenic mouse models tend to fall into one of three major categories – modeling Aβ plaque formation, Tau hyper-phosphorylation, and a combination of these two key AD hallmarks. Depending on the mutations/copy number involved, each mouse model shows pathological features at different ages and to varying intensity. Additionally, other secondary characteristics such as synaptic loss, neuroinflammation, and cognitive deficits are often detectable in transgenic mouse models of AD.

The first two major transgenic mouse models of AD were produced in the mid-1990s. The PDAPP(line 109) was produced by inserting the human APP gene encoding an

APP mutation, V717F (Indiana) under a platelet-derived growth factor β (PDGF-β) promoter. The PDGF-β promoter is expressed in brain, fat, lung and thyroid (Fagerberg et al., 2014). These mice express three splicing variants of human APP (695, 751, and 770) (Games et al., 1995) which may better recapitulate relevant pathologies seen in human forms of AD. The PDAPP mouse model of AD exhibits strong Aβ deposition around 4 months old (mo) and cognitive impairments at 6 mo (Games et al., 1995) (**Table 1.2**).

The next major transgenic mouse model of AD was the Tg2576 model that overexpressed a human double mutation of APP (APP K670N/M671L (Swedish)) under the hamper prion protein promoter. This transgenic mouse line expresses APP isoform 695 developing extensive amyloid pathology and cognitive deficits at older ages (Hsiao et al., 1996) relative to PDAPP (**Table 1.2**). The slower onset of disease may better model AD as the disease's greatest risk factor is aging.

In the early 2000's, another aggressive mouse model of AD was developed that combined multiple familial mutations. The APP/PS1 mouse model combines the same Swedish mutation seen in Tg2576, but adds an additional PS1 mutation first observed in two families with early-onset familial AD (Perez-Tur et al., 1995). These two transgenes are integrated together under the control of the mouse prion promoter (Jankowsky et al., 2001). The PS1 mutation is missing exon 9 (ΔE9) from mRNA transcripts, and therefore, mice with this mutation produce more Aβ42, which is known to aggregate more aggressively than other common Aβ species (Jarrett & Lansbury, 1993). The APP/PS1 line forms plaque pathology around 6 mo and behavioral deficits by 7-9 mo (**Table 1.2**)

The 3xTg mouse line attempted to recreate both pathological features of AD (plaques and tangles) into one mouse model. The 3xTg line gets its name from combining three familial AD mutations (APP KM670/671NL Swedish, MAPT P301L, and PSEN1 M146V) (Oddo et al., 2003). The Microtubule-associated protein tau (MAPT) mutation P301L within exon 10 causes accelerated formation of paired helical filaments seen in AD (Barghorn et al., 2000). Therefore, these mice present both plaque and tangle pathology starting around 4 mo (**Table 1.2**). As seen in human AD progression, hyperphosphorylated tau aggregates are detected in the hippocampus after amyloid deposits are already established.

Next, to investigate the role of tau hyperphosphorylation alone, a mouse model was made using the same MAPT P301L mutation as in the 3xTg line, but absent of all plaque-forming mutations. Regulation of this inserted MAPT overexpression is under the neuron-specific mouse Thy1 promoter (Terwel et al., 2005). After mice have reached ~8 mo, tau tangles begin to form primarily in the brainstem and spinal cord (**Table 1.2**). Tangles continue to accumulate to a maximum of 12 mo, when affected mice die prematurely. Therefore, this mouse model is not ideal for extensive long-term age-related pathologies. Additionally, chronic drug treatments would have to be planned to start/end at younger ages than would be required of most other AD models.

The 5xFAD (Tg 6779) mouse model is the one that exhibits extremely aggressive AD-related phenotypes at a young age (**Table 1.2**). Genetically, this mouse was produced with five early-onset familial AD mutations (APP KM670/671NL (Swedish), APP I716V

(Florida), APP V717I (London), PSEN1 M146L (A>C), PSEN1 L286V). Statistically speaking, it would be highly improbable for any individual to possess all five mutations that are expressed in these mice. However, they are still a useful tool to study plaque formation on accelerated timelines. Additionally mouse lines with medium (Tg7031) and low (Tg 7092) expression levels were also developed which show the same pathological hallmarks but at a delayed rate of onset (Oakley et al., 2006). Importantly, these mice have been crossed with targeted replacement apoE2 (E2FAD), apoE3 (E3FAD), and apoE4 (E4FAD) mice to study the interplay between plaque formation and its dependency and susceptibility to different apoE genotypes (Youmans et al., 2012). Note that apoE4 is highly associated with sporadic onset of AD and all mouse models referenced thus far have focused on familial mutations.

Table 1.2 - Abbreviated list of commercially available mouse models of Alzheimer's disease:

Mouse Line	Genes Expressed	Plaque Formation	Tangle Pathology	Cognitive Impairment	Synaptic Loss	Source Reference
PDAPP	APP V717F (Indiana)	6 mo	NA	3-9 mo	<6 mo	(Games et al., 1995)
Tg2576	APP KM670/671NL (Swedish)	12 mo	NA	6 mo	4.5 mo	(Hsiao et al., 1996)
APP/PS1	APP KM670/671NL (Swedish), PSEN1: deltaE9	6 mo	NA	8 mo	4 mo	(Jankowsky et al., 2001)
3x Tg	APP KM670/671NL (Swedish), MAPT P301L, PSEN1 M146V	6 mo	12 mo	4 mo	NA	(Oddo et al., 2003)
Tau P301L	MAPT P301L	NA	8 mo	6 mo	NA	(Terwel et al., 2005)
5xFAD	APP KM670/671NL (Swedish), APP I716V (Florida), APP V717I (London), PSEN1 M146L (A>C), PSEN1 L286V	1.5 mo	NA	4-5 mo	4-5 mo	(Oakley et al., 2006)

Connections between Alzheimer's disease, lipoproteins, and cardiovascular disease (Hottman et al., 2014):

Lipoproteins, as the name implies, are made of a combination of several types of lipids and proteins that form a hydrophobic core and a hydrophilic shell (Rosenson et al., 2011). They are formed in extracellular space and circulate as soluble subcellular-sized particles in body fluids. The main function of lipoproteins is facilitating the delivery and clearance of lipids and lipid-soluble or associating molecules throughout the body. The hydrophobic core contains neutral lipids, predominantly triglycerides (TG) and cholesterol esters (CE). The hydrophilic shell consists of primarily phospholipids (PL), unesterified free cholesterol (FC), and various apolipoproteins, which mediate interactions with a variety of other molecules including enzymes, transporters, and receptors through a dynamic process. One critically important molecule in a variety of cellular functions is cholesterol. FC is synthesized in the mevalonate pathway. There are several other compounds produced in the mevalonate pathway such as heme A, dolichol, and ubiquinone. It is also important to note that several key hormones such as progesterone, aldosterone, and corticosterone are further products of mevalonate pathway (**Fig. 1.1**). Lipoproteins are separated and categorized based on their density into six sub-fractions (from low to high-density): chylomicrons, very-low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and lipoprotein (a) (LP(a)) (Nikolic et al., 2013). Low HDL-C and high LDL-C levels are strongly implicated in cardiovascular disease (Stampfer et al., 1991).

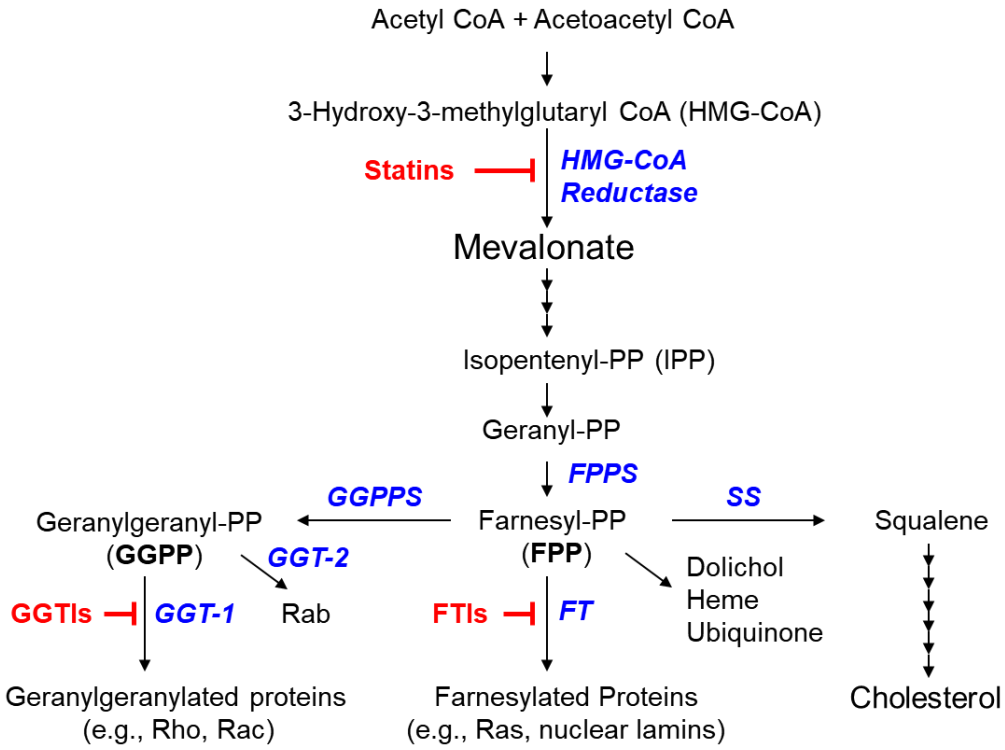

Fig. 1.1 – The mevalonate pathway. HMG-CoA reductase is a rate-limiting enzyme in the mevalonate pathway. Statins inhibit the activity of HMG-CoA reductase and limit the production of isoprenoid intermediates and cholesterol. Farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) serve as lipid donors for protein prenylation. They are also both important precursors to cholesterol. FPP is a major branching point in the cholesterol synthesis pathway. It is a precursor to all protein prenylation but also dolichol, heme, ubiquinone, squalene which eventually leads to cholesterol.

Several lines of evidence suggest that AD and cardiovascular disease share common risk factors and pathogenic mechanisms. Recent reports correlate low levels of HDL with the onset of cognitive decline in AD patients (Merched et al., 2000). Additionally, elevated HDL has been associated with healthy aging. Interestingly, AD patients with high levels of HDL exhibit less severe AD-related symptoms than patients with lower HDL levels (Merched et al., 2000). Several genetic and environmental factors contribute to HDL levels and function. Of interest, apolipoprotein (apo) E4 carriers have a higher incidence of hypercholesterolemia and atherosclerosis (Corder et al., 1993). Additionally, the apoE4 allele is present in nearly half of AD patients (Corder et al., 1993).

These patients have elevated low-density lipoproteins (LDL) cholesterol, increasing the risk of developing coronary artery disease (CAD) and exacerbated levels of Aβ. Experimentally, diet-induced hypercholesterolemia causes Aβ deposits in the brain of rabbits (Sparks et al., 1994) and accelerates cerebral Aβ deposition in APP transgenic mice (Refolo et al., 2000; Levin-Allerhand et al., 2002; Shie et al., 2002). Finally, our previous research has shown that diet-induced and LDL receptor deficiency-induced hypercholesterolemia exacerbates amyloid pathology and memory deficits in a transgenic mouse model of AD (Li et al., 2003; Cao et al., 2007).

In humans, cholesteryl ester transfer protein (CETP) activity mediates the transfer of cholesteryl esters from HDL to other lipoproteins, resulting in reduced HDL-cholesterol levels. Recent genomics studies have identified several mutations within the CETP gene. Mutations that resulted in reduced CETP activity have been associated with elevated plasma HDL-cholesterol levels and linked to healthy aging (Nir Barzilai et al., 2003). Interestingly, mice do not have CETP or an analogous protein (Guyard-Dangremont et al., 1998; Haa and Barter 1982). Transgenic expression of CETP in mice lowers HDL-cholesterol levels (Jiang et al., 1992).

Apolipoprotein A-I (apoA-I) is a 243-residue protein primarily produced in the liver and intestine. ApoA-I is a core protein component of plasma HDL-cholesterol and determines the function of HDL. Strong evidence establishes that plasma apoA-I/HDL inversely correlate with the risk of cardiovascular disease (Davidson and Toth 2007). AD patients exhibit reduced plasma apoA-I (Johansson et al., 2017), and reduced plasma apoA-

I is highly correlated with AD symptom severity (Merched et al., 2000). Therefore, raising HDL levels or improving HDL function is becoming an attractive therapeutic target for both cardiovascular disease and AD. We have recently shown that the overexpression of apoA-I in a mouse model of AD doubled plasma HDL-cholesterol levels (Lewis et al., 2010). This increase in apoA-I/HDL resulted in reduced neuroinflammation and CAA without lowering total Aβ deposition (Lewis et al., 2010). CAA is caused by local Aβ deposition in cerebral vessels which reduces nutrient delivery to the brain. Increased CAA resulted in reduced spatial learning and exacerbated memory deficits. Conversely, decreasing apoA-I results in exacerbated AD pathology (Lefterov et al., 2010). Clinically, statins are used to decrease plasma LDL cholesterol and have been associated with a reduced risk of developing AD (Jick et al., 2000). Together, these results strongly implicate the importance of cholesterol in the pathogenesis of AD.

Role of high-density lipoprotein in Alzheimer's disease (Hottman et al., 2014):

High-density lipoprotein metabolism in the systemic circulation:

Lipoproteins may be characterized by their size, density, electrophoretic mobility, and composition. The most commonly used classification of lipoproteins is by density. Due to the dynamic nature of the lipoproteins, each class of lipoproteins can be divided into several subclasses. Specifically, HDL has a density of 1.063 – 1.210 g/mL and size of approximately 7-20 nm. They are formed both in the systemic circulation and in the brain. Plasma HDL has been studied extensively because of its well-established protective role in the cardiovascular system. Recent studies strongly suggest that the benefits of HDL

extend to the central nervous system (CNS). Mounting evidence indicates that HDL modulates cognitive function in aging and age-related neurodegenerative disorders.

Although HDL is often referred to as HDL cholesterol (HDL-C), apoA-I is the major protein component of HDL in the plasma and determines most of its functions (Segrest et al., 2000). The lipid-associating domain (residues 44-243) of human apoA-I contains tandem repeats of amphipathic α-helixes (Segrest et al., 1992). HDL biogenesis starts with the interaction between lipid-poor apoA-I and ATP-binding cassette transporter A1 (ABCA1) on the cell membrane of peripheral tissues, resulting in the formation of nascent discoidal HDL particles from cell membrane-derived PL and FC (Oram and Heinecke 2005). Of note, other apolipoproteins can also act as lipid acceptors for ABCA1. Importantly, this is the first step of RCT, a process that removes excess cholesterol from peripheral tissues to the liver for excretion in the bile. Once the discoidal particles reach plasma, apoA-I activates lecithin cholesterol acyltransferase (LCAT), forming mature, spherical, CE-rich HDL particles (**Fig. 1.2**).

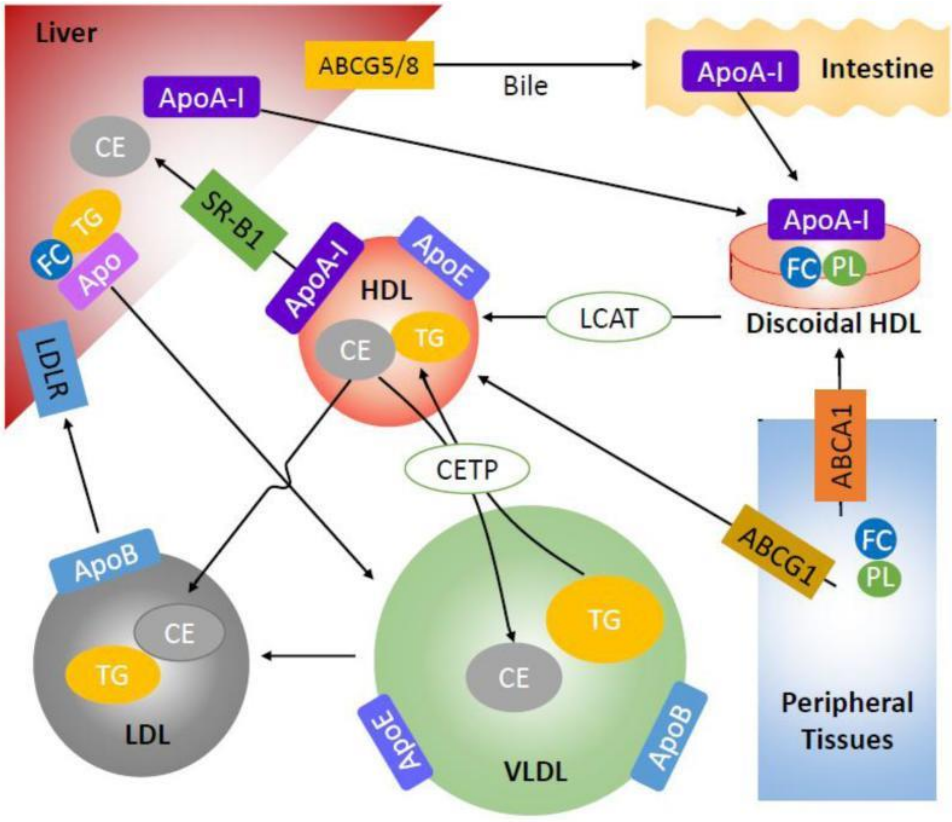

Fig. 1.2 – Schematic of HDL metabolism in the systemic circulation. Formation of the nascent discoidal HDL through apoA-I and ABCA1 is the first step in reverse cholesterol transport (RCT), a process that removes excess cholesterol from peripheral tissues to the liver for excretion. In the plasma, apoA-I activates LCAT, which converts discoidal HDL to mature, spherical, CE-rich HDL particles. HDL interacts with other lipoprotein particles and cells through multiple receptors, transporters, and enzymes. Mature HDL removes cholesterol from peripheral cells through other ABC transporters such as ABCG1. Lipid-rich HDL selectively delivers CE to hepatocytes and steroidogenic cells through SR-B1. HDL-bound CETP mediates the exchange of CE from HDL to non-HDL particles and the transfer of TG from TG-rich lipoproteins to HDL.

In the plasma, HDL interacts with cells and other lipoprotein particles through multiple receptors, transporters, and enzymes. Mature HDL can remove cholesterol from peripheral cells through other ABC transporters ABCG1/ABCG4, further promoting RCT. Lipid-rich HDL selectively delivers CE to hepatocytes and steroidogenic cells through scavenge receptor B1 (SR-B1), regenerating lipid-poor apoA-I/HDL particles for further interaction with ABCA1. HDL-bound CETP mediates the exchange of CE from HDL to non-HDL particles and the transfer of TG from TG-rich lipoproteins to HDL, resulting in

decrease of HDL-C levels. Thus, CETP inhibitors have been developed to raise HDL levels (discussed below). Other major HDL-interacting proteins include phospholipid transfer protein (PLTP), endothelial lipase, and hepatic lipase (Vance and Vance 2008).

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

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

al., 2011). Clearly, the complexity of HDL composition and function presents both the challenge and opportunity to develop HDL-based biomarkers and therapies for a number of diseases.

High-density lipoprotein metabolism in the central nervous system:

While lipoprotein metabolism in the systemic circulation has been studied extensively, interest in lipoprotein metabolism in the brain has only increased in recent years because of connections between apoE and the development of several neurological disorders. The brain is highly enriched in cholesterol and relies on *de novo* synthesis since cholesterol does not cross the blood-brain barrier (BBB)(Zhang & Liu, 2015). The CNS contains ~25% of total body cholesterol despite the fact that the brain accounts for only 2% of total body mass (Dietschy and Turley 2001). In adults, the rate of cholesterol synthesis exceeds the need for forming new structures. One of the excretory pathways involves the formation of 24S-hydroxycholesterol that crosses the BBB into the plasma (Dietschy and Turley 2001).

The major apolipoprotein in the brain is apoE, which is primarily produced by glial cells. In humans, there are three isoforms of apoE coded by three alleles: *APOE*-ε2, *APOE*-ε3, and *APOE*-ε4, with an allele frequency of 7%, 78%, and 15%, respectively (Strittmatter and Roses 1996). ApoE has received tremendous attention due to its genetic association with AD. While the *APOE*-ε2 allele confers some protection against AD (Corder et al., 1994), the *APOE*-ε4 allele is associated with an increased risk of AD (Corder et al., 1993; Poirier et al., 1993). The brain also expresses lipoprotein receptors (e.g., LDLR, LRP, and

SR-B1), enzymes (e.g., LCAT and lipases), transfer proteins (e.g., PLTP and CETP), and ABC transporters (e.g., ABCA1 and ABCG1), although the presence of CETP in the brain is controversial (Albers et al., 1992; Demeester et al., 2000; Yamada et al., 1995). Because these proteins have well-established roles in cholesterol metabolism in the periphery, they are thought to play similar functions in the brain (**Fig. 1.3**).

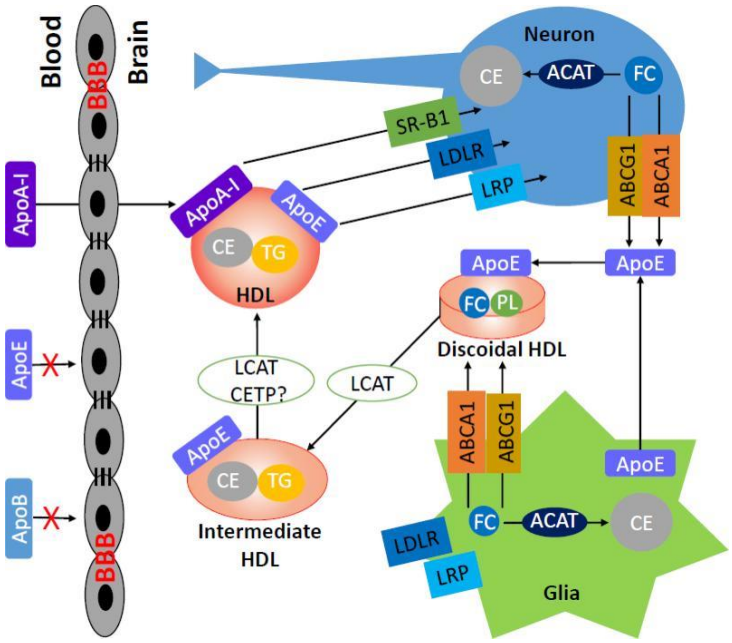

Fig. 1.3 – Schematic of HDL metabolism in the brain. Similar to the peripheral tissues, the brain expresses the various lipoprotein receptors (e.g., LDLR, LRP, and SR-B1), enzymes (e.g., LCAT and lipases), transfer proteins (e.g., PLTP and CETP), and ABC transporters (e.g., ABCA1 and ABCG1), although the presence of CETP in the brain is controversial. ApoE synthesized primarily by glia and apoA-I from the blood generate HDL particles and mediate cholesterol efflux through interactions with ABCA1 and ABCG1. LCAT converts the discoidal HDL to mature HDL particles. The HDL particles are remodeled by the interactions of apoE and apoA-I with various lipoprotein receptors on neurons and glia.

HDL-like lipoprotein particles are found in the CSF and contain mainly apoE and apoA-I (Koch et al., 2001; Ladu et al., 2000). While the source of apoE is clearly from glia as plasma apoE cannot cross the BBB (Linton et al., 1991), the origin of apoA-I in the CSF is uncertain. It is generally thought that the brain does not produce apoA-I and that apoA-I in the brain comes from the circulation (Dietschy and Turley 2001). However, porcine

cerebral endothelial cells have been shown to produce apoA-I (Möckel et al., 1994). Notably, the concentration of apoA-I in the CSF is comparable to that of apoE (Koch et al., 2001). In addition, plasma and CSF HDL cholesterol and apoA-I levels are correlated, suggesting that plasma apoA-I/HDL levels can influence brain apoA-I/HDL levels (Fagan et al., 2000). While the role of apoE in brain cholesterol metabolism and other pathways is well established (Yu, Youmans, and LaDu 2010), the neurobiological role of apoA-I has not been well studied. Experimental evidence has shown that rat astrocytes interact with both human apoE and apoA-I and generate HDL-like particles with distinct properties: apoE-HDL particles are cholesterol-rich whereas apoA-I-HDL particles are phospholipid-rich (Ito et al., 1999). Human CSF lipoproteins are capable of inducing a significant cholesterol efflux from rat astrocytes (Demeester et al., 2000). The efflux ability of CSF lipoproteins is correlated more with the concentration of apoA-I in the CSF than that of apoE (Demeester et al., 2000). Also, exogenous human apoA-I is able to initiate a signal transduction pathway of intracellular cholesterol trafficking involving the activation of protein kinase C (PKC) in rat astrocytes for HDL biogenesis (Ito et al., 2002; Ito et al., 2004). In addition, apoA-I and apoE-containing HDL in the CSF go through different remodeling in response to traumatic brain injury in human (Kay et al., 2003). These findings, together with other evidence discussed below, suggest that apoA-I-containing HDL may have important functions in the brain under physiological and pathological conditions.

High-density lipoprotein and age-related cognitive decline:

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

Notably, the genotype of apoE, known as a major genetic determinant for AD, also affects cognitive decline in normal aging. Carriers of the *APOE*-ε4 allele showed decline in memory before the age of 60 years and exhibited greater acceleration than non-carriers (Caselli et al., 2009). A recent study also showed that aging individuals without dementia, but with possession of the *APOE*-ε4 allele have a higher rate of cognitive decline in the ninth decade of life (Schiepers et al., 2012). It is also worth noting that carriers of *APOE*-ε4 have a proatherogenic lipid profile with lower HDL and higher VLDL and TG levels in the plasma than non-carriers. A recent study suggests that the high lipid affinity of apoE4 is responsible for such a lipid profile (Hui Li et al., 2013). These findings suggest that besides the direct influence of apoE4 on brain function, systemic effects of apoE4 may also contribute to the compromised cognitive performance in carriers.

In addition to the *APOE*-ε4 allele, recent gene association studies provided further evidence for the beneficial effects of HDL and/or apoA-I on cognitive decline in aging. Functional polymorphisms in the gene for CETP, which cause lower levels of CETP and higher levels of HDL, are associated with slower cognitive decline in aging (Barzilai et al., 2006; Sanders et al., 2010; Izaks et al., 2012), although some inconsistency exists (Yu et al., 2012). Furthermore, genetic variants in apoC-III, which cause lower levels of triglycerides and higher levels of HDL, are associated with exceptional longevity (Atzmon et al., 2006) and cardioprotection (Pollin et al., 2008; Jørgensen et al., 2014; Crosby et al., 2014), whereas the apoC-III variants with opposite effects are associated with impaired cognition (Smith et al., 2009). Whole-genome sequence-based analysis suggests that common variation contributes more to heritability of HDL levels than rare variation

(Morrison et al., 2013). Whether all HDL-regulating genetic variations affect cognitive function awaits further investigation.

High-density lipoprotein and Alzheimer's disease:

The pathogenesis of AD, particularly the sporadic form of AD, is not fully understood. While aging itself is the biggest risk factor for AD, the *APOE*-ε4 allele is a major genetic risk factor for sporadic AD (Corder et al., 1993) and the role of apoE in AD has been well studied. In addition to *APOE*, recent large genome-wide association studies have identified over 20 loci that contribute to the risk of sporadic AD (reviewed in (Rosenthal and Kamboh 2014; Reitz 2012)). Several loci, such as *CLU* (clusterin or apoJ) and *ABCA7*, are closely involved in the cholesterol metabolism pathway. However, both clusterin and ABCA7 also have roles in the innate immunity. Whether CLU and ABCA7 variants associated with the AD risk influence brain or plasma HDL levels or functions is unknown.

Clinical studies in different ethnic populations have shown that high levels of plasma HDL were associated with a decreased risk for AD, although there have been a few exceptions (Launer et al., 2001; Reitz et al., 2004; Vollbach et al., 2005). An early study with a group of 45 Japanese patients with AD found that plasma levels of apoA-I and apoA-II were markedly decreased compared to 79 controls (Kawano et al., 1995). Consistently, a study with a cohort of 98 French AD patients and 59 controls showed that decreased HDL cholesterol and serum apoA-I concentrations were highly correlated with the severity of AD (Merched et al., 2000). Another study with 334 elderly French subjects found that high

HDL cholesterol levels were associated with a significantly decreased risk of AD (Bonarek et al., 2000). Furthermore, the Honolulu-Asia aging study with 929 men indicated that the levels of apoA-I and HDL cholesterol were inversely associated with the risk of AD (Saczynski et al., 2007). More recently, the Manhattan cognitive study with 1,130 individuals also showed that high levels of HDL cholesterol were associated with a decreased risk of both probable and possible AD (Reitz et al., 2010). Consistently, the InChianti study with 1,051 Italians older than 65 years of age reported that low HDL cholesterol levels were associated with dementia (Zuliani et al., 2010). In addition, another recent study with 664 subjects from the Sydney Memory and Aging study reported that elderly individuals with MCI had abnormal plasma levels of HDL-associated apolipoproteins. MCI subjects had lower levels of apoA-I, apoA-II and apoH, and higher level of apoE and apoJ. Lower apoA-I, apoA-II and apoH levels increased the risk of cognitive decline over two years. Intriguingly, among the apolipoproteins, apoA-I was the most significant predictor of cognitive decline (Song et al., 2012).

Further support for a protective role of HDL in AD comes from studies in animal models. Generally, mice are not the ideal animal model for studying human lipoprotein metabolism and AD due to physiological differences between the species. However, many different transgenic mouse models have been developed to mimic relevant human physiology (Laferla and Green 2017; Getz and Reardon 2012). Multiple laboratories have consistently shown that genetic and pharmacological manipulation of important players in HDL biogenesis-related pathways, such as ABCA1 and liver X receptors (LXR), modifies the development of AD-like pathology and cognitive impairment in mouse models of AD

(Burns et al., 2006; Fitz et al., 2010; Donkin et al., 2010; Jiang et al., 2008; Hirsch-Reinshagen et al., 2005; Koldamova et al., 2005; Koldamova et al., 2005; Riddell et al., 2007; Vanmierlo et al., 2011; Wahrle et al., 2005; Zelcer et al., 2007; Wesson et al., 2011). Furthermore, genetic overexpression of human apoA-I and accompanied increase of functional HDL prevented the development of age-related cognitive deficits in the APP/PS1 mouse model of AD (Lewis et al., 2010). Consistently, lack of apoA-I exacerbated cognitive deficits in APP/PS1 mice (Lefterov et al., 2010). Intriguingly, genetic manipulation of apoA-I does not affect total brain parenchymal Aβ deposition (Fagan et al., 2004; Lewis et al., 2010; Lefterov et al., 2010) but significantly changes the dynamics of cerebrovascular Aβ deposition; apoA-I overexpression attenuates whereas apoA-I deficiency exacerbates CAA in AD mice (Lefterov et al., 2010; Lewis et al., 2010). Notably, HDL deficiency could be particularly detrimental in *APOE*-ε4 carriers as a recent study showed that ABCA1 deficiency worsened AD-like cognitive impairment and Aβ deposition in human apoE4 but not in apoE3-targeted replacement mice. In apoE4 mice, plasma HDL and Aβ levels were significantly decreased and the plasma HDL level was negatively correlated with amyloid plaques in the brain, suggesting a role of plasma HDL in Aβ clearance (Fitz et al., 2012). Taken together, these findings provide compelling evidence that HDL and associated apolipoproteins play a pivotal role in modulating the pathogenesis of AD.

Potential mechanisms by which high-density lipoprotein modulates cognitive function:

Although the evidence for the protective role of HDL in cognition is substantial, the underlying mechanisms by which HDL modulates cognitive function are poorly understood. Clearly, multiple functions of HDL are involved under different conditions. To simplify the discussion, AD is used to illustrate potential mechanisms of action for apoA-I to modulate the disease process (**Fig. 1.4**). Since the systemic effects of HDL are well established (Davidson and Toth 2007) and the cerebrovascular function of HDL in AD has been summarized recently by an excellent review (Stukas et al., 2014), this section focuses on the potential direct role of apoA-I in the brain.

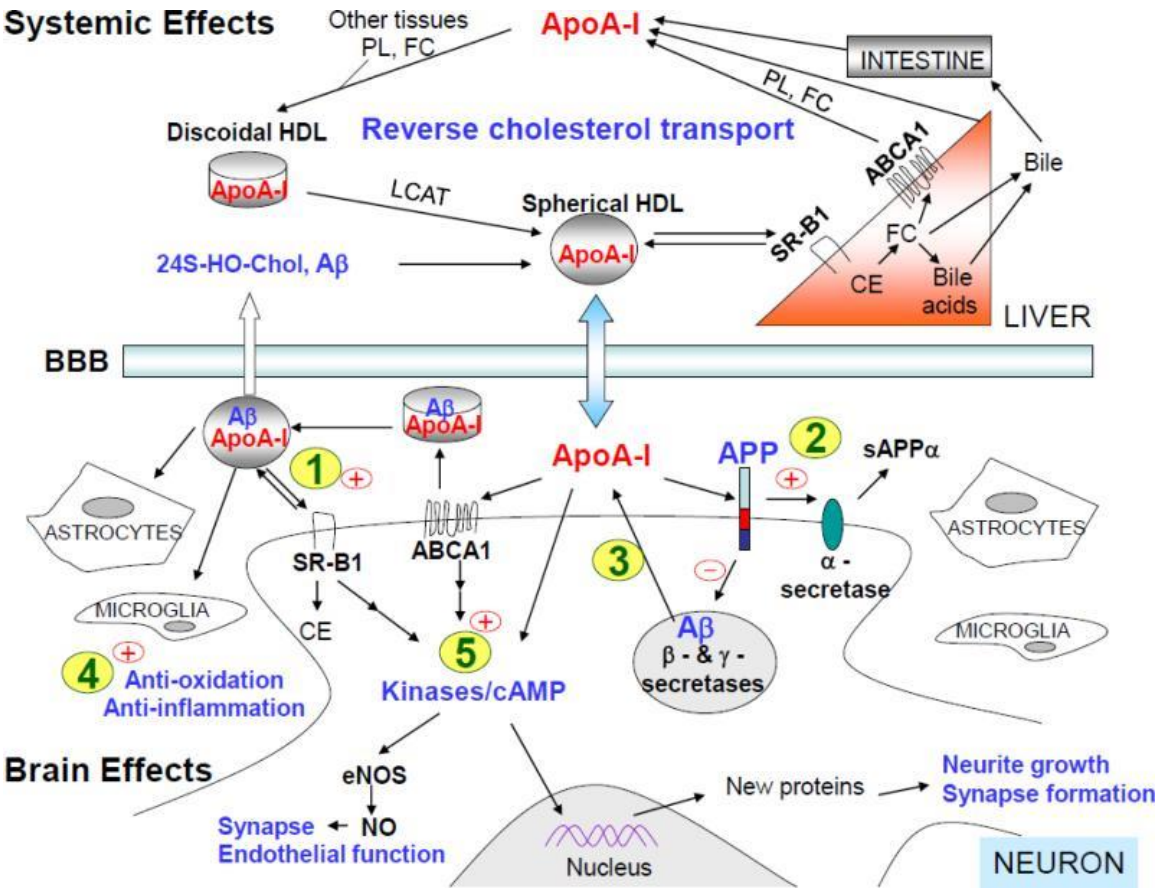

Fig. 1.4 – Schematic of potential mechanisms by which apoA-I and HDL modulates AD pathogenesis. ApoA-I is hypothesized to act on 5 major pathways to exert its neuroprotective effects pertinent to AD. (1) Cholesterol efflux pathway. ApoA-I in the brain promotes the cellular cholesterol efflux through ABCA1 and forms HDL-like particles. These particles are cleared by interacting with receptors such as SR-B1 by cells in the brain or through the BBB to peripheral circulation. (2) APP trafficking and processing pathway. ApoA-I-mediated changes in membrane fluidity may enhance α-secretase cleavage of APP at the cell membrane. Also, apoA-I binds to the extracellular domain of APP, which may prevent APP from undergoing the endocytic process, thereby inhibiting the access of β- and γ-secretases to get access to APP and reducing the generation of Aβ. (3) Aβ clearance pathway. ApoA-I binds to Aβ and inhibits Aβ aggregation. ApoA-I/HDL in the brain can mediate the clearance of Aβ by local cells (e.g., astrocytes and microglia) through the scavenger receptor (e.g., SR-B1) and/or by crossing the BBB to the systemic circulation. (4) Anti-oxidation and anti-inflammation. ApoA-I/HDL possesses anti-oxidant and anti-inflammatory properties that are neuroprotective. (5) Signal transduction and synaptic plasticity. ApoA-I/HDL activates several kinases and increase the level of cAMP directly or indirectly through ABCA1 or SR-B1. These molecules play important roles in signaling pathways pertinent to synaptic function and memory formation.

Cholesterol efflux pathways: It has been shown *in vitro* and *in vivo* that, as in the periphery, apoA-I in the brain promotes the cellular cholesterol efflux through ABCA1 and forms discoidal HDL-like particles (Ito et al., 1999; Wahrle et al., 2004). With the

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

Amyloid precursor protein trafficking and processing pathways: ApoA-I/HDL in the brain may affect the APP processing pathways through both of the following mechanisms: a) apoA-I mediates efficient cellular cholesterol efflux (Demeester et al., 2000) the resultant increase in membrane fluidity could enhance α-secretase cleavage of APP at the cell membrane and b) apoA-I binds to APP at the cell surface (Koldamova et al., 2001); thereby it may prevent APP from undergoing the endocytic process, which is necessary for β- and γ-secretases to get access to APP. Thus, the final consequence of these effects would be less generation of Aβ.

Amyloid β clearance pathways: Overproduction of Aβ in the brain causes familial AD, but impaired Aβ clearance from the brain is implicated in sporadic AD (Scheuner et al., 1996; Castellano et al., 2011; Mawuenyega et al., 2010). ApoA-I binds to Aβ and inhibits Aβ aggregation and cytotoxicity *in vitro* (Koldamova et al., 2001). In addition, the

binding affinity of human apoA-I for Aβ is higher than that of human apoE (Koldamova et al., 2001). Therefore, the apoA-I/HDL in the brain is expected to be more effective in binding Aβ and mediates the clearance of Aβ by local cells (e.g., astrocytes and microglia) through the scavenger receptor (e.g., SR-B1) and/or by crossing the BBB to the systemic circulation (Sagare et al., 2012). Supporting this notion, studies in APP/PS1 mice have demonstrated that lack of apoA-I exacerbates whereas overexpression of human A-I ameliorates cerebrovascular deposition of Aβ (Lefterov et al., 2010; Lewis et al., 2010). Additionally, a recent study has shown that apoE has minimal direct interaction with Aβ and competes with Aβ for the same clearance pathways within the brain (Verghese et al., 2013). These intriguing results suggest that upregulation of apoA-I and/or inhibition of apoE competition with Aβ for cellular uptake in the brain might be an effective means to enhance Aβ clearance.

Anti-oxidation and anti-inflammation pathways: Oxidative stress and inflammation contribute to the etiology of AD (Keeney et al., 2013; Schrag et al., 2013; Wyss-Coray and Rogers 2012). Anti-oxidant and anti-inflammatory properties of apoA-I/HDL have been shown to play significant roles in protecting against cardiovascular disease (Barter et al., 2004). These same mechanisms may play a significant role in neuroprotection. Previous studies support this hypothesis: a) the level of CSF apoA-I is increased significantly after infection in macaques (Saito et al., 1997); b) CSF apoA-I-containing lipoproteins remodel after traumatic brain injury in humans (Kay et al., 2003); c) reconstituted human apoA-I-containing HDL reduces neuronal damage in rat models of stroke, via an anti-oxidative mechanism (Paternò et al., 2004); d) an apoA-I mimetic peptide inhibits inflammation in

the brain and improves cognitive performance in mice (Buga et al., 2006; Handattu et al., 2009); and e) overexpression of human apoA-I attenuates neuroinflammation in AD mice (Lewis et al., 2010).

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

Potential of high-density lipoprotein–enhancing pharmacotherapies to improve cognitive function:

All five currently available FDA approved therapies for AD treat symptomatic aspects of the disease and not the underlying dysfunctions. Fortunately, there are several novel mechanisms, targets, and compounds aimed at reducing Aβ burden and preventing AD progression.

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

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

Niacin has been used for over 50 years to raise HDL-C levels (Carlson 2005). At present, niacin is the most effective HDL-raising agent available clinically. It also lowers the level of TG, lipoprotein (a), and LDL-C (Toth et al., 2013). A recent clinical trial (AIM-HIGH; Clinicaltrials.gov NCT00120289) showed that in patients with cardiovascular disease and low HDL-C levels, treatment with extended-release niacin, 1500 to 2000 mg per day, significantly increased HDL-C (25%) while decreasing TG (29%) and LDL-C (16%) (McBride 2011). Further analysis also showed that niacin treatment modestly

increased apoA-1 (7%), decreased apoB (13%), decreased the apoB/apoA-I ratio (19%), and decreased Lipoprotein (a) (21%) (Albers et al., 2013). However, these favorable changes in lipoprotein profiles did not lead to the reduction of cardiovascular events (Boden et al., 2011). It is worth noting that the patients in this trial were receiving intensive statin therapy and their baseline LDL-C was very low (74 mg/dL) (McBride 2011). Thus, it is possible that no additional benefits from niacin treatment can be achieved in patients with very low LDL-C levels. This possibility is supported by another recent clinical trial (HPS2-THRIVE ClinicalTrials.gov Identifier: NCT00461630) (Haynes et al., 2013). In this study, participants were treated with extended-release niacin combined with laropiprant, a prostaglandin-D2 receptor-1 inhibitor, to alleviate niacin-induced facial flushing. Subgroup analysis from this study showed that in participants with LDL-C lower than 78mg/dL no benefit was found with niacin/laropiprant treatment, but in participants with LDL-C higher than 78 mg/dL benefit was observed with the treatment (Haynes et al., 2013). It is worth noting that the formulation of niacin influences the side effects of niacin. Standard immediate-release niacin causes a high frequency of flushing and long-acting niacin causes less flushing but increases the risk of hepatotoxicity, whereas extended-release niacin causes fewer of both types of adverse effects (McKenney 2003). Thus, a proper formulation of niacin should be selected to reduce potential side effects of long-term use of niacin.

In addition to niacin, synthetic GRP109A agonists, such as MK-1903, have been developed. MK-1903 has been evaluated in phase I and II studies to treat dyslipidemia. MK-1903 treatment produced a significant decrease in plasma free fatty acids. However,

MK-1903 had a smaller effect on serum lipid levels compared with niacin, suggesting that niacin may act on a GRP109A-independent pathway (Boatman et al., 2012). Further studies in animal models and humans confirmed that GPR109A receptor does not mediate niacin's lipid efficacy (Lauring et al., 2012), opening the door for identifying new molecular target (s) of niacin and developing novel approaches to raise HDL.

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

In addition, fibrates are commonly used in combination therapy with statins. It is important to note that the combination of gemfibrozil and statin significantly increases the risk of rhabdomyolysis (Pierce et al., 1990; Staffa et al., 2002), due to partial inhibition of gemfibrozil on the metabolism of statins (Prueksaritanont et al., 2002). In contrast to gemfibrozil, fenofibrate does not increase the concentrations of statins (Bergman et al., 2004). The combination of fenofibrate and statin has been used in large, long-term clinical trials and there was no evidence for an increased risk of myositis or rhabdomyolysis compared to statin monotherapy (Ginsberg et al., 2010; Farnier et al., 2011). A recent meta-analysis on the safety of the co-administration of statin with fenofibrate also concluded that statin-fenofibrate combination therapy was tolerated as well as statin monotherapy (Guo et al., 2012).

Statin monotherapy: Atorvastatin is a commonly prescribed 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor to treat hypercholesterolemia and interest arose when long-term statin use was thought to be protective against AD (Shepardson et al., 2011). However, the LEADe trial (ClinicalTrials.gov Identifier: NCT00024531) recruited 641 patients with mild to moderate AD and tested to see if there was any cognitive benefit to an 18 month treatment with 80mg/day atorvastatin (Jones et al., 2008). Unfortunately, there was no cognitive benefit in mild to moderate AD patients who were randomized to atorvastatin (Feldman et al., 2010). However, smaller scale future studies did report positive effects on cerebral blood flow in patients taking atorvastatin (Xu et al., 2008). It is possible that early interventions would be more beneficial in preventing initial cognitive decline in patients at risk for developing AD.

Similar to atorvastatin, simvastatin is an HMG-CoA reductase inhibitor to treat hypercholesterolemia and was FDA approved in 1991 (Cechinel-Filho 2012). Original excitement around statins in AD arose from a six-month clinical trial conducted in Germany, which reported that simvastatin treatment lowered Aβ CSF levels in AD patients (Simons et al., 2002; Locatelli et al., 2002). However, additional small-scale follow-up studies have been less clear (Hoglund et al., 2005). In 2003, a 406 patient multicenter study was performed to evaluate if simvastatin was able to slow AD progression. The CLASP trial (ClinicalTrials.gov Identifier: NCT00053599) reported expected changes in cholesterol levels, but did not alter cognitive function or cognitive decline (Sano et al., 2011). However, it has been speculated that while simvastatin is incapable of restoring memory function, it may be protective if started prior to AD symptom onset. To address this question, the multicenter SIMaMCI (Clinical Trials.gov NCT00842920) study recruited 445 patients with memory impairment, but without diagnosed AD. The SIMaMCI study plans to determine if simvastatin can delay the time until patients convert from MCI to AD. The study is set to run through 2019 and is expected to provide crucial insight into mechanisms underlying dementia conversion.

Apolipoprotein A-1 infusion: The strong negative correlation between plasma apoA-I levels and cardiovascular disease and consistent experimental results in animal models have led to direct infusion of apoA-I in human clinical trials. Nissen et al., infused human patients with a recombinant apoA-I_{Milano}, a form of apoA-I that is associated with lower risk for cardiovascular disease (Franceschini et al., 1980), and observed a significant regression of coronary atherosclerosis (Nissen et al., 2003). Another group performed a

randomized human trial to test the infusion of apoA-I incorporated into recombinant HDL (rHDL). The study determined that short-term infusion of rHDL produced a significant reduction of atheroma volume and improved plaque characterization index and coronary score (Tardif et al., 2007). More evidence of atheroprotection was obtained with the infusion of apoA-I or apoA-I_{Milano} in both animal models and human clinical trials. Remarkably, a single infusion was shown to be enough to significantly reduce atherosclerosis and infer positive effects on plaque characterization (Tardif 2010). Additionally, researchers infused rabbits with either lipid free apoA-I or apoA-I in rHDL. The infusions markedly inhibited vascular inflammation in the rabbits (Patel et al., 2010). A recent study showed that infusion of apoA-I produced an increase in cholesterol efflux from macrophages, favorably remodeled HDL and reduced cytokine secretion in both rabbits and human blood (Diditchenko et al., 2013).

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

Apolipoprotein A-I and high-density lipoprotein mimetic peptides: A major obstacle in the path of using native apoA-I as therapeutics is its lack of oral bioavailability.

Additional concern stems from the high cost and relative difficulty of mass-producing full-length apoA-I. Thus, the development of orally bioavailable small peptides, which retain the atheroprotective effects of apoA-I, was a highly enticing prospect. These small peptides are described as apoA-I mimetics. The general design of the apoA-I mimetics is an amphipathic peptide, which adopts an alpha helical secondary structure similar to that seen in the full-length apoA-I (Segrest et al., 1992). Mimetics can be synthesized from D-amino acids and thus have higher oral bioavailability. Of note, in addition to apoA-I mimetics, peptides derived from other HDL-associated apolipoproteins including apoE and apoJ have also been created. Readers interested in gaining a more comprehensive understanding of HDL mimetic peptides are encouraged to refer to excellent recent reviews (Getz et al., 2010; Leman et al., 2014).

Research into the development of apoA-I mimetic peptides began in an effort to design therapeutics for atherosclerosis. In line with that goal, a number of mimetic peptides were created and tested for therapeutic benefit in mice and cell culture. In order to preserve lipid-binding and anti-atherosclerotic activity, an 18 amino acid peptide was designed without sequence homology to apoA-I, but remaining structurally similar. The peptide, called 18A, formed an amphipathic alpha helical secondary structure and was shown to have a similar lipid-binding capacity as full-length apoA-I (Anantharamaiah et al., 1985).

Modifications were made to 18A, wherein a number of non-polar residues were replaced with phenylalanines (F) in an attempt to bolster its atheroprotective affects, and the most successful of these modified peptides was 4F (Datta et al., 2001). The oral

bioavailability of 4F in the plasma was quite low, however, and so its enantiomer, D-4F, was created and shown to remain in the plasma for much longer after oral gavage (Navab et al., 2005). The atheroprotective and anti-inflammatory efficacy of 4F has been described *in vitro*, in animal models and in human clinical trials. D-4F has been shown to inhibit atherosclerotic lesion development and also to reduce inflammation in mice and rabbits (Navab et al., 2005; Van Lenten et al., 2007). The D-4F peptide promotes RCT, induces functional changes in macrophage activity, and reduces lipid oxidation in vascular plaques (Smythies et al., 2010; Navab et al., 2005). Furthermore, a single dose of D-4F was well tolerated and improved the HDL anti-inflammatory profile of human patients with cardiovascular disease (Bloedon et al., 2008). These data make further studies on D-4F a particularly intriguing objective.

Due to the known correlation between vascular risk factors and cognitive decline, HDL mimetic peptides have been tested for efficacy in improving mental health. In fact, D-4F has been shown to have effects on cognitive capacity. In LDL receptor-null mice, D-4F was shown to reduce inflammation in the vasculature of the brain and improve cognitive performance without influencing plasma lipid levels (Buga et al., 2006). Additionally, D-4F in combination with pravastatin was shown to inhibit Aβ plaque formation and improve cognitive function by inducing an anti-inflammatory effect in the brain without affecting plasma HDL-C levels (Handattu et al., 2009), suggesting that D-4F improves the quality not the quantity of HDL and/or directly modulate disease-related processes in the brain.

Cholesteryl ester transport protein inhibitors: Based on several lines of evidence that CETP deficiency/inhibition is associated with an elevated level of HDL and a decreased risk for cardiovascular disease, CETP inhibitors have been developed and tested in clinical trials. Torcetrapib was the first CETP inhibitor tested. In ILLUMINATE trial (ClinicalTrials.gov Identifier: NCT00134264) (Barter et al., 2007), torcetrapib significantly increased the level of HDL-C in treated patients but failed to show a clinical benefit. In fact, torcetrapib was associated with an increase in cardiovascular events due to unexpected off-target adverse effects resulting in hypertension. Dalcetrapib was the second CETP inhibitor to undergo clinical trials. In the dal-OUTCOMES trial (ClinicalTrials.gov identifier NCT00658515) (Schwartz et al., 2012), dalcetrapib successfully increased HDL-C levels but did not reduce recurrent cardiovascular events. Dalcetrapib was safe and the reason for its failure is not clear. It has been suggested that dalcetrapib-induced increase in HDL-C levels might not have been sufficient or it was not accompanied by an enhancement of the protective properties of HDL (Rader and deGoma 2014; Toth et al., 2013). Two new CETP inhibitors, anacetrapib and evacetrapib, are much more potent than dalcetrapib and do not have the off-target adverse effects of torcetrapib (Gotto and Moon 2012; Nicholls et al., 2011). However, similar to the dal-OUTCOMES trial, outcomes from the ACCELERATE evacetrapib trial (ClinicalTrials.gov Identifier: NCT01687998) were disappointing. Evacetrapib successfully elevated HDL and reduced LDL, but these changes did not result in a significantly reduction in the incidence of cardiovascular events compared to placebo among patients with high-risk vascular disease (Riesmeyer et al., 2017; Eyvazian and Frishman 2017). Encouragingly, the anacetrapib REVEAL trail

(ClinicalTrials.gov Identifier: NCT01252953) met its primary endpoint of significantly reducing major coronary events compared to placebo (Landray 2017). Full results will be available later in 2017. Additionally, the DEFINE trial (ClinicalTrials.gov Identifier: NCT00685776) is a smaller scale trial with anacetrapib aimed at ensuring there are no adverse cardiovascular events that were seen with torcetrapib and full results will be available later in 2017. Interim analysis showed similar findings to the larger REVEAL trial (ClinicalTrials.gov Identifier: NCT01252953) (Brinton et al., 2015).

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

Several synthetic LXR agonists, including T0901317, GW3965 and LXR-623, are currently undergoing experimental testing for the treatment of dyslipidemia and atherosclerosis. Recently, accumulating preclinical evidence indicates the therapeutic potential of LXR agonists for AD. Studies in multiple laboratories have shown that LXR agonists improve cognitive functions either with or without reducing Aβ levels in the brain of AD mice (Donkin et al., 2010; Fitz et al., 2010; Jiang et al., 2008; Riddell et al., 2007;

Vanmierlo et al., 2011; Wesson et al., 2011). Specifically, in the APP23 mouse model of AD, T0901317 treatment ameliorated amyloid pathology and memory deficits (Fitz et al., 2010). It was shown that T0901317 treatment resulted in a decrease in Aβ levels in the interstitial fluid of the hippocampus, supporting the role of LXR agonists in facilitating Aβ clearance. *In vitro* experiments demonstrated that ABCA1 was essential for lipidation of apoE and mediated the effects of T0901317 on Aβ degradation by microglia (Fitz et al., 2010). The specific role of ABCA1 in mediating benefits of LXR agonists in AD mice was further confirmed by another study with GW3965 in the APP/PS1 mouse model of AD (Donkin et al., 2010). These findings indicate that LXR agonists exert neurological benefits through the ABCA1/apoE-HDL pathway. Interestingly, a recent study showed that GW3965 treatment dramatically increased the level of apoA-I in the brain of APP/PS1 mice independent of ABCA1 (Stukas et al., 2012). Therefore, increase of apoA-I/HDL may also contribute to the beneficial effects of LXR agonists in AD mice.

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

of AD after as few as 7 days of treatment (Cramer et al., 2012). However, the effectiveness of bexarotene in AD mice has been questioned by subsequent studies as the reduction of Aβ plaques in treated mice could not be reproduced (Tesseur et al., 2013; Veeraraghavalu et al., 2013; Price et al., 2013; Fitz et al., 2013). The discrepancy observed in these studies might result from differences in drug formulations and mouse models (Landreth et al., 2013). Nevertheless, some studies replicated the decrease in soluble Aβ levels (Fitz et al., 2013; Veeraraghavalu et al., 2013) and the improvement of cognitive function (Fitz et al., 2013; Tesseur et al., 2013) in bexarotene-treated AD mice. Importantly, bexarotene increased Aβ clearance and rescued cognitive function in APP/PS1 mice expressing either human apoE3 or apoE4 isoform (Fitz et al., 2013). In contrast, a more recent study did not find any changes in Aβ plaques or cognitive deficits in bexarotene-treated APP/PS1 mice (LaClair et al., 2013). Thus, further studies are required to clarify the effects of bexarotene on AD-related processes.

Apolipoprotein A-1 upregulators – RVX-208: RVX-208 is a novel small molecule that stimulates apoA-I gene expression leading to an increase in HDL levels and functionality (Bailey et al., 2010). Recent studies showed that RVX-208 is a specific inhibitor for BET bromodomains that regulate expression of a variety of genes including apoA-I (McLure et al., 2013; Picaud et al., 2013). Early testing in African green monkeys demonstrated that a 63-day RVX-208 treatment markedly increased serum apoA-I (60%) and HDL-C levels (97%), accompanied by the enhancement of ABCA1, ABCG1, and SR-B1-mediated cholesterol efflux (Bailey et al., 2010).

Positive results from animal models and early human clinical trials have led to further human clinical trials. The Phase IIb SUSTAIN trial (ClinicalTrials.gov Identifier: NCT01423188) was designed to evaluate the lipid efficacy, safety and tolerability of RVX-208, and the ASSURE trial (ClinicalTrials.gov Identifier: NCT01067820) was designed to evaluate the effect of RVX-208 on atherosclerotic plaque burden using intravascular ultrasound (IVUS) imaging (Nicholls et al., 2012). Findings from these clinical trials suggest that RVX-208 has the potential for the treatment of cardiovascular disease.

In addition, RVX-208 may also have a therapeutic potential for diabetes and AD. A Phase II clinical trial of RVX-208 in pre-diabetic patients is ongoing (ClinicalTrials.gov Identifier: NCT01728467). On AD, a pilot Phase Ia study showed a trend of increase in the level of Aβ40 in the plasma of patients treated with RVX-208 for 7 days compared to controls (Resverlogix 2008)(http://www.resverlogix.com/upload/latest_news/81/01/2008-11-10_alzheimers_program_final.pdf). This preliminary result was confirmed in a more recent Phase II ASSERT trial (ClinicalTrials.gov Identifier: NCT01058018), in which 12-week treatment with RVX-208 significantly increased the plasma Aβ40 level compared to baseline or the level of placebo-treated controls (Resverlogix 2011) (http://www.resverlogix.com/media/press-release.html?id=451). These intriguing observations are consistent with the findings from genetic upregulation of apoA-I in AD mice (Lewis et al., 2010) and support the notion that elevating apoA-I levels in the systemic circulation enhances Aβ clearance from the brain. Further clinical trials of RXV-208 in AD or pre-AD patients will be needed to determine whether RVX-208 can modulate the progression of AD.

Protein prenylation in relation to synaptic function (Hottman & Li, 2014):

Isoprenoids and protein prenylation:

As noted previously, cholesterol shares a synthesis pathway with several other important biological processes. One such pathway includes the production of intermediary isoprenoids, which can also be pharmacologically manipulated with statins. In particular, the isoprenoid farnesyl-pyrophosphate (FPP) is a major branching point in the mevalonate pathway between further isoprenoid/prenylation targets and cholesterol synthesis. Levels of FPP and geranylgeranyl pyrophosphate (GGPP) are elevated in AD patients indicating a 'back up' in the normal synthesis pathway (Eckert et al., 2009). Indeed, alteration of protein prenylation has been implicated in many types of cancers (Sebti & Hamilton, 2000; Whyte et al., 1997) and viral infection (Ye et al., 2003).

Many proteins undergo posttranslational modifications that allow for proper protein folding, trafficking, and function (Krishna and Wold 1993). These modifications often include the addition of functional groups such as phosphates, lipids and carbohydrates. The functions of proteins are regulated by posttranslational modifications. One type of lipid posttranslational modifications is prenylation (Lane and Beese 2006). Prenylation refers to the addition of short-chain lipid molecules called isoprenoids to the C-terminus of target proteins. Isoprenoids are short-chain lipid molecules formed in the mevalonate pathway for cholesterol biosynthesis (Goldstein and Brown 1990) (**Fig. 1.5**). These lipid attachments facilitate the anchoring of proteins to the cell membrane and mediate protein-protein interactions. Prenylated proteins are involved in regulating a variety of cellular

functions including synaptic plasticity and in the pathogenesis of a number of diseases including AD.

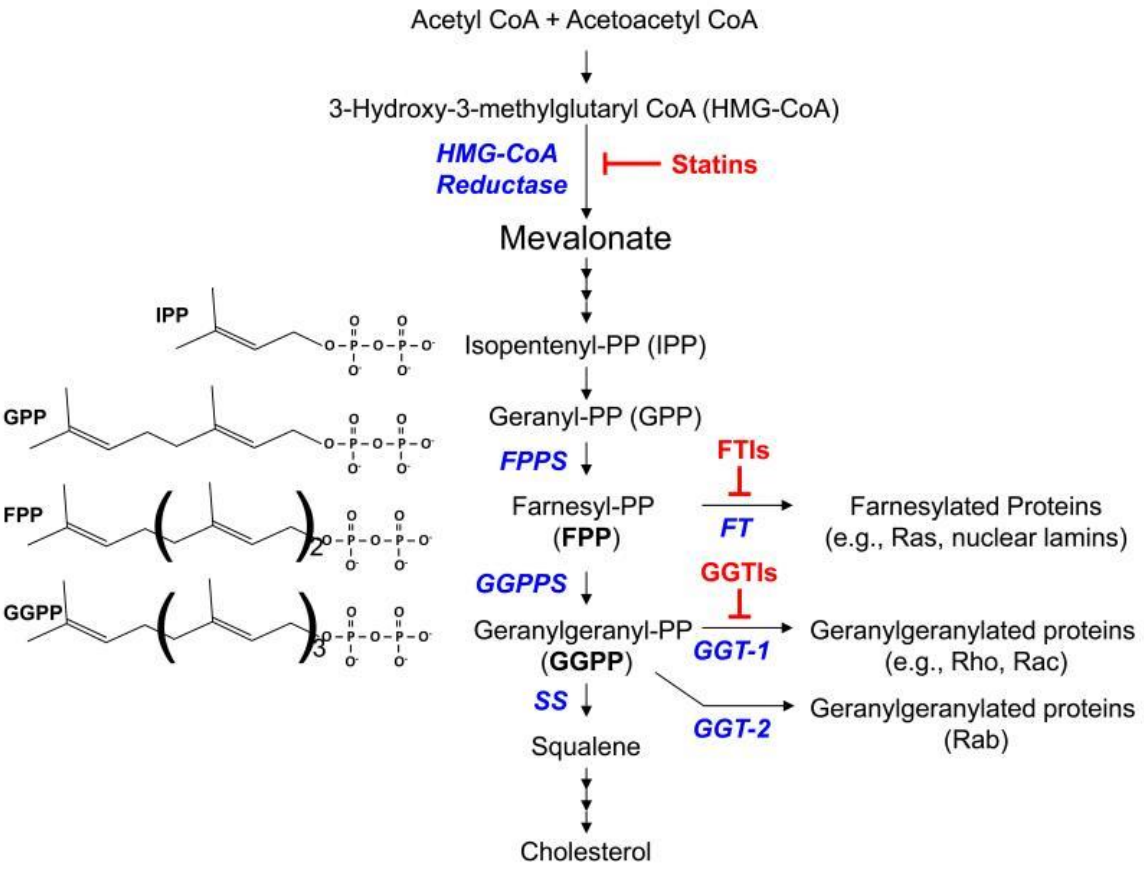

Fig. 1.5 – The mevalonate pathway focusing on isoprenoids. HMG-CoA reductase is a rate-limiting enzyme in the mevalonate pathway. Statins inhibit the activity of HMG-CoA reductase and limit the production of isoprenoid intermediates and cholesterol. Farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) serve as lipid donors for protein prenylation. Farnesyl transferase inhibitors (FTIs) and geranylgeranyl transferase-1 inhibitors (GGTIs) block protein farnesylation and geranylgeranylation, respectively.

The 15-carbon isoprenoid, FPP, is a major branching point in the mevalonate pathway that is commonly associated with cholesterol synthesis. FPP serves as a substrate for several enzymes, including squalene synthase (SS), farnesyl transferase (FT), and GGPP synthase (GGPPS) that produces the 20-carbon GGPP. FPP is also a precursor for

the synthesis of long-chain isoprenoids such as dolichol, ubiquinone (coenzyme Q), and heme. FPP and GGPP serve as lipid donors for protein prenylation.

During protein farnesylation and geranylgeranylation, collectively called protein prenylation, FPP and GGPP are covalently attached to the C-terminus of target proteins, respectively (Lane and Beese 2006). Farnesylation is catalyzed by protein farnesyl transferase (FT) and occurs on cysteine residues present in tetrapeptide recognition sequences (CAAX, in which C is cysteine, A is an aliphatic amino acid and X is variable) located at the C-termini of their target protein substrates. In contrast, geranylgeranylation is catalyzed by two different protein geranylgeranyl transferases. Geranylgeranyl transferase-1 (GGT-1) acts on substrates that contain C-terminal tetrapeptide sequences similar to but distinct from FT substrates, whereas geranylgeranyl transferase-2 (GGT-2 or RabGGT) recognizes more structurally complex sequences and exclusively prenylates Rab proteins (Lane and Beese 2006; Leung et al., 2006). Protein prenylation is an important posttranslational modification that allows proteins to anchor to the cell membrane or other subcellular locations and mediates protein-protein interactions (McTaggart 2006).

Statins are drugs commonly used to regulate blood cholesterol levels. Stains work by inhibiting HMG-CoA reductase, a rate-limiting step in the mevalonate pathway for cholesterol biosynthesis that converts of acetyl-CoA to mevalonate (**Fig. 1.2** and **1.5**). Inhibition of HMG-CoA reductase results in a decreased level of FPP and GGPP, and thus, may lead to decreased farnesylation and geranylgeranylation of proteins (Liao 2002; Vaughan 2003). As such, it is difficult to dissect the roles of farnesylation and

geranylgeranylation using statins. To this end, drugs have been developed to specifically target FT and GGT-1 (Berndt et al., 2011). Farnesyl transferase inhibitors (FTIs) and geranylgeranyl transferase-1 inhibitors (GGTIs) are powerful tools for studying the function of specific prenylation pathways. These drugs are currently under investigation for the treatment of cancers and other disorders (Li et al., 2012).

Over 100 proteins are known to undergo prenylation (McTaggart 2006; Berndt et al., 2011). They include heterotrimeric G protein subunits and nuclear lamins but the largest and most extensively studied group is the Ras superfamily of small GTPases.

Ras superfamily of small GTPases:

The Ras GTPase superfamily consists of over 150 known members, divided between five major subfamilies: Ras, Rho, Rab, Arf/Sar and Ran (Rodriguez-Viciana et al., 2004). Specific small GTPases regulate a number of effector proteins and may have different final intracellular locations, and in some cases, differential prenylation can affect the subcellular distribution and function of small GTPases (Du et al.,1999; Liu et al., 2000).

In general, small GTPases act as molecular switches that are activated, or 'turned on' by guanine nucleotide exchange factors (GEFs) and inhibited, or 'turned off' by GTPase-activating proteins (GAPs) (Klooster 2007; Tolias and Duman 2011). GEFs promote the dissociation of guanosine diphosphate (GDP) from small GTPases (Schmidt et al., 2002). This dissociation step allows the exchange of GDP for guanosine triphosphate (GTP). A small GTPase is considered 'active' when GTP is bound. Antagonistically, GAPs enhance the rate of the weak intrinsic GTP hydrolysis activity of small GTPases (Bernards

and Settleman 2004). Small GTPases depend on prenylation for proper cellular localization and function (McTaggart 2006). Inhibiting small GTPase prenylation affects many cellular functions such as cytoskeletal stability and the efficiency of vesicular transport (Ridley 2001). More recent studies have also revealed that the interplay between small GTPase GEFs and GAPs regulates spine morphogenesis and synapse development (Kiraly et al., 2010). Interestingly, GGT-1 itself has been shown to have a direct role in neuromuscular junction formation and maintenance by controlling acetyl choline receptor rearrangement during development (Luo et al., 2003). In addition to morphological changes, small GTPases are involved in multiple signaling pathways that regulate synaptic plasticity (Ye and Carew 2010).

Synaptic plasticity:

In its most general definition, synaptic plasticity is the strengthening or weakening of the synapse between two neurons over time (Hughes 1958). Synaptic plasticity has many underlying mechanisms, including pre-synaptic changes regulating the amount of neurotransmitter released, and post-synaptic changes such as the incorporation of new neurotransmitter receptors (Gerrow and Triller 2010). In general, both the strengthening and weakening of a synapse depends on calcium uptake. Two major molecular mechanisms of synaptic plasticity in the hippocampus include the regulation and activity of N-methyl D-aspartate (NMDA) and α-amino-3-hydroxy-5methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors (Shi et al., 1999) (**Fig. 1.6**).

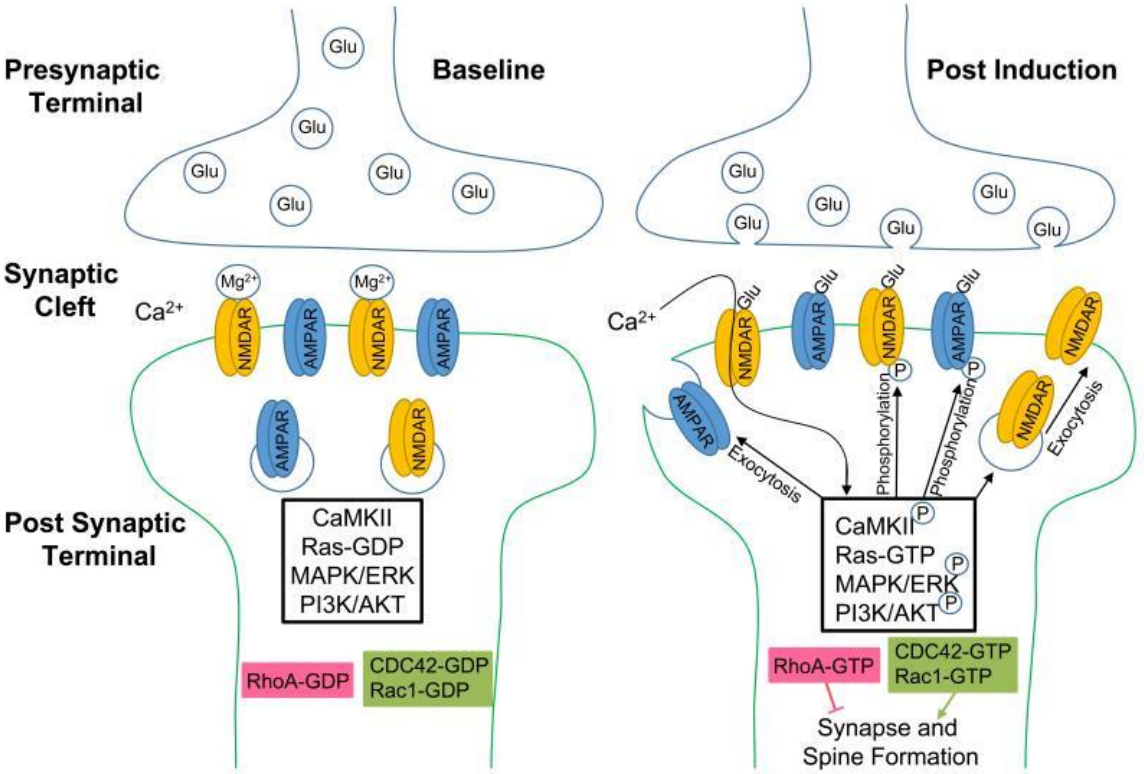

Fig. 1.6 – Schematic diagram of a synapse. At resting membrane potentials, NMDA receptors are blocked by Mg^{2+}. Low intracellular concentrations of Ca^{2+} prevent the autophosphorylation of Ca^{2+}/calmodulin-dependent protein kinase (CaMKII). B) Post-synaptic depolarization pushes the Mg^{2+} out of the NMDA channel pore, allowing Ca^{2+} to enter the cell. Glutamate (Glu) from the pre-synaptic cell binds to AMPA and NMDA receptors to depolarize the post-synaptic cell. The rise in intracellular Ca^{2+} promotes CaMKII autophosphorylation. Once phosphorylated, CaMKII phosphorylates AMPA subunits to enhance their conductance. CaMKII also promotes the exocytosis of receptor containing vesicles and thereby increases the presence of receptors at the synapse. Furthermore, CaMKII activates RasGEFs (guanine exchange factors) and promotes the turnover of inactive Ras-GDP to active Ras-GTP. Downstream signaling cascades include mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT). When activated (phosphorylated), ERK and AKT facilitate exocytosis of glutamate receptors during synaptic plasticity. In addition, neuronal activity activates Rho GTPases. Active (GTP-bound) Rac1/Cdc42 enhance while RhoA inhibits synapse/spine formation.

Notably, before NMDA receptors open their ion channel, glutamate must be present at the receptor while the post-synaptic cell is depolarized (Dingledine et al., 1999). This is due to a magnesium block in the NMDA channel pore that must be expelled by a reduction in the voltage across the post-synaptic cell membrane (Nowak et al., 1984). Once open, NMDA channels allow for calcium to rush into the cell and trigger downstream signaling

cascades that alter the synaptic strength between the two cells (Nowak et al., 1984; Premkumar and Auerbach 1996). These NMDA receptors act as a 'coincidence detector' because two events need to occur within a narrow temporal window to allow calcium to flow into the post-synaptic cell (Caporale and Dan 2008).

Calcium influx from NMDA receptors is necessary for the activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Lisman 1994). The activation of NMDA receptors is a mechanism to transfer the information of mutual depolarization across the synapse. If the two cells continue to fire strongly together, their synapses will strengthen over time by the addition of AMPA and NMDA receptors to the post-synaptic cell membrane, or phosphorylation of the subunits of existing AMPA and NMDA receptors in the membrane (Barria et al., 1997; Mammen et al., 1997). The changes in synaptic potentiation exist in equilibrium, and therefore, AMPA and NMDA receptors can be removed/dephosphorylated from synapses.

Synaptic plasticity can be divided into two major categories, short-term plasticity, and long-term plasticity. Long-term plasticity can be further separated into long-term depression (LTD), early long-term potentiation (E-LTP), and late long-term potentiation (L-LTP) (Sweatt 1999; Kandel 2001; Malenka and Bear 2004). LTD is the weakening of synaptic strength over time, often by removal or dephosphorylating of post-synaptic receptors. E-LTP is the enhancement of synaptic strength that is not dependent on protein synthesis. L-LTP has the longest lasting effects, and is dependent on synthesis of new proteins in response to increased synaptic activity. Small GTPases are involved in

regulating multiple aspects of synaptic plasticity and the following sections will briefly discuss the roles of Ras and Rho subfamilies of proteins in synaptic function.

Ras and synaptic plasticity:

Ras subfamily GTPases were originally studied for their role in oncogenesis. Constitutively active mutations in Ras small GTPases occur in 8% to 93% of cancers depending on the tumor type (Bos 1989). This underscores the importance of proper regulation of small GTPases. Normally, Ras small GTPases play crucial roles in regulating cell proliferation, differentiation, cell survival, and memory formation (Konstantinopoulos et al., 2007; Ye and Carew 2010). The well-known members of Ras subfamily of small GTPases include H-Ras, K-Ras, and N-Ras. Ras small GTPases primarily undergo farnesylation but some of them can also undergo geranylgeranylation. For example, while H-Ras is exclusively farnesylated, K-Ras and N-Ras can be geranylgeranylated when farnesyltransferase is inhibited (Liu et al., 2010). Major downstream signaling cascades of Ras include mitogen-activated protein kinases (MAPK), such as extracellular signal-regulated kinase (ERK), and phosphoinositide 3-kinase (PI3K), which regulate glutamate receptor trafficking during synaptic plasticity (**Fig. 1.6**) (Ye and Carew 2010; Stornetta and Zhu 2010).

Several lines of evidence indicate that H-Ras plays a negative role in regulating synaptic plasticity and memory function. In a mouse model of neurofibromatosis type 1 mental retardation, which is characterized by the hyperactivity of Ras, learning/memory and synaptic function are severely impaired (Costa et al., 2002; Li et al., 2005). Treatment

with an FTI or a statin rescues hyperactive Ras-induced synaptic and memory impairment (Costa et al., 2002; Li et al., 2005). Similarly, H-Ras overexpression negatively affects the NMDA receptor transmission by decreasing the level of tyrosine phosphorylation of the NMDA receptor NR2A subunit (Thornton et al., 2003). In contrast, mice deficient in H-Ras expression display enhanced tyrosine phosphorylation of NMDA receptors and NMDA receptor-mediated hippocampal LTP (Manabe et al., 2000). These data support the role of Ras small GTPases in regulating NMDA receptor dependent synaptic plasticity.

Rho and synaptic plasticity:

The Rho subfamily of GTPases primarily undergoes geranylgeranylation, although some are exclusively farnesylated (e.g., RhoE). Others, such as RhoB, can be either farnesylated or geranylgeranylated (Adamson et al., 1992; Baron et al., 2000). Interestingly, geranylgeranylated and farnesylated RhoB exhibit distinct and opposite functions. Geranylgeranylated RhoB inhibits cell growth, whereas farnesylated RhoB promotes cell growth and transformation (Du et al., 1999; Liu et al., 2000). However, for some Rho GTPases, although their functions depend on being prenylated, either geranylgeranylated or farnesylated form works equally. For example, RhoA is exclusively geranylgeranylated under physiological conditions; when RhoA is mutated to become susceptible to farnesylation, the farnesylated RhoA shows similar subcellular location and functions as the geranylgeranylated RhoA (Solski et al., 2002).

The Rho subfamily of GTPases is one of the major regulators in synaptic plasticity, both in dendrite morphogenesis and stability as well as in growth cone motility (Govek et

al., 2011; Newey et al., 2005; Tolias and Duman 2011). Rho proteins are well documented for their role in the regulation of actin rearrangement in neuronal cytoskeletons. Specifically, three major Rho proteins, RhoA, Rac1, and Cdc42, regulate neuronal structures and synaptic connectivity (Newey et al., 2005; Govek et al., 2011; Tolias and Duman 2011) . When activated by GEFs, Rho small GTPases interact with effector proteins, initiating signaling cascades that control actin cytoskeletal rearrangement, microtubule rearrangement, transcription, membrane trafficking, and act as key regulators of dendritic growth and spine morphogenesis (Govek et al., 2011; Newey et al., 2005; Tolias and Duman 2011) (**Fig. 1.6**).

The interaction between the Rho GTPases determines the complexity of the dendritic tree and the formation of spines. RhoA, Rac1, and Cdc42 play differential roles in regulating dendritic growth and spine formation. Activation of Rac1 and Cdc42 promotes dendritic branching/remodeling and spine formation, whereas activation of RhoA exhibits opposite function, reducing dendritic growth/complexity and spine density/length (Newey et al., 2005). Rac1 is highly expressed in the hippocampus of adult mice (Tejada-Simona et al., 2006). The hippocampus is well known for its synaptic plasticity and its importance in developing associative memories (Gruart et al., 2006; Kandel 2001). Rac1 plays an important role in the formation of neuronal synapses at their correct locations. Specifically, *in vitro* studies show that NMDA receptor activation induces membrane translocation and activation of Rac1 in the CA1 region of the hippocampus (Tejada-Simona et al., 2006). Activation of tyrosine kinase receptor B (TrkB) by brain-derived neurotropic factor (BDNF) leads to the activation of Rac1 and induces changes in cellular

morphology (Miyamoto et al., 2006). Notably, BDNF-dependent dendritic morphogenesis requires the activation of GGT-1, the enzyme that catalyzes the geranylgeranylation of Rac1 and other Rho proteins (Zhou et al., 2008). In addition, TrkB is physically associated with GGT-1 and neuronal activity enhances this association and GGT-1 activity, further promoting dendritic spine morphogenesis (Zhou et al., 2008). Conversely, activation of RhoA inhibits dendritic growth and spine formation in multiple model systems (Newey et al., 2005). The negative role of RhoA on dendritic growth and spine morphogenesis is partly mediated by the RhoA effector Rho-kinase (ROCK) (Nakayama et al., 2000). Specific inhibitors of ROCK can block active RhoA-induced dendritic simplification (Nakayama et al., 2000). The balance between the positive and negative effects of Rac1/Cdc42 and RhoA guarantees the proper development of dendrites and dendritic spines that are important postsynaptic structures regulating synaptic plasticity.

Implications of protein prenylation in Alzheimer's disease:

In the brain of AD patients, Aβ accumulates as the disease progresses. The structural integrity of synapses degrades rapidly during β-amyloidosis (Klyubin et al., 2012), with the longer amyloidogenic Aβ42 being more potent than Aβ40 in disrupting synaptic plasticity (Nomura et al., 2012). One of the mechanisms by which Aβ impairs synaptic function is by promoting endocytosis of NMDA receptors and thereby reducing the presence of NMDA receptors at the cell surface (Snyder et al., 2005). Importantly, the impairment of synaptic function in the hippocampus occurs prior to the appearance of insoluble amyloid plaques and neuronal cell death (Selkoe et al., 2002). However,

inhibition of Aβ-producing enzymes under normal conditions results in abnormalities in synaptic function (Wang et al., 2012). These findings suggest that Aβ itself may have normal physiological functions which are disrupted by abnormal accumulation of Aβ during AD pathology.

Emerging evidence indicates that isoprenoids/protein prenylation and small GTPases affect multiple aspects of AD (**Fig. 1.7**) (Cole and Vassar 2006; Hooff et al., 2010).

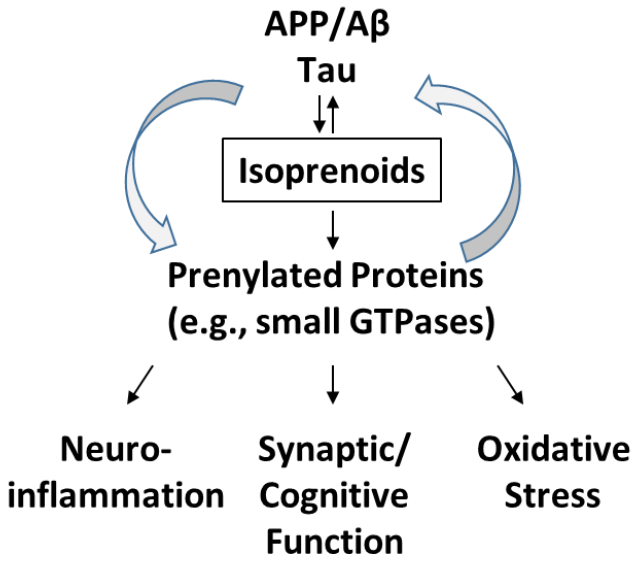

Fig. 1.7 – Schematic diagram of interplay between prenylated proteins and AD pathology.

For example, statin-induced depletion of isoprenoids leads to reduced levels of protein prenylation and promotes non-amyloidogenic processing of APP and reduces the production of Aβ (Pedrini et al., 2005; Cole et al., 2005; Ostrowski et al., 2007; Zhou et al., 2008). Interestingly, while geranylgeranylated RhoA-mediated activation of ROCK increases Aβ secretion via modulation of γ-secretase (Zhou et al., 2003), specific inhibition

of farnesylated RhoB/ROCK pathway promotes α-secretase activity (Pedrini et al., 2005). Of note, although inhibitors of ROCK reduce total Aβ secretion, targeting ROCK by expression of dominant-negative or constitutively active ROCK mutants failed to modulate Aβ secretion (Leuchtenberger et al., 2006). Additional *in vitro* experiments show that statin-induced low isoprenoid conditions cause the accumulation of intracellular APP, the C-terminal fragment of APP produced by β-secretase cleavage (β-CTF), and Aβ, which can be rescued by GGPP supplementation, suggesting the involvement of geranylgeranylated target proteins (Cole et al., 2005). The study also shows that low isoprenoid levels inhibit the trafficking of APP through the secretory pathway (Cole et al., 2005). A more recent study further demonstrates that low isoprenoid conditions induced by physiologically relevant doses of statins preferentially inhibit the geranylgeranylation of Rab family proteins involved in vesicle trafficking and thereby affects the trafficking and intracellular localization of APP (Ostrowski et al., 2007). Inhibition of Rac also regulates APP expression and processing (Wang et al., 2009; Boo et al., 2008). In contrast, supplementation of FPP and/or GGPP stimulates the production of Aβ (Zhou et al., 2008, 2003; Kukar et al., 2005).

Intriguingly, the interplay between isoprenoids/prenylated proteins and APP/Aβ metabolism appears to be reciprocal. It has been shown that Aβ and other APP cleavage products such as APP intracellular domain (AICD) may directly regulate the activities of the enzymes in the mevalonate pathway thereby changing the levels of isoprenoids and other lipids (Grimm et al., 2005, 2012). Consistent with these findings, the levels of FPP and GGPP are elevated in the brains of patients with AD (Eckert et al., 2009), suggesting

that the abundance of prenylated proteins could be increased in AD brains. Indeed, the level of Ras (both cytosolic and membrane/prenylated fractions) in the brain is increased in the early stage of AD (Gärtner et al., 1995, 1999), suggest that upregulation of Ras may play an important role in the pathogenic cascade leading to AD. Aβ causes cellular dislocation and dysfunction of Rac and its effector protein PAK (Zhao et al., 2006; Ma et al., 2008). Also, the level of prenylated RhoA is increased in Aβ-treated neuroblastoma cells and in the neurons surrounding Aβ plaques in AD mice (Petratos et al., 2008). Conversely, a recent study shows that a toxic level of oligomeric Aβ42 inhibits protein prenylation (Mohamed et al., 2012).

In addition to APP/Aβ metabolism, prenylation/GTPases have been shown to be involved in other aspects of AD pathology. For instance, inhibition of prenylation of Rho GTPases leads to attenuation of Aβ-induced neuroinflammation (Cordle and Landreth 2005; Cordle et al., 2005). Limiting the availability of isoprenoids for prenylation has been shown to protect neurons from Aβ-induced apoptosis via activating pro-survival signaling pathways (Johnson-anuna et al., 2005; Franke et al., 2007; Cespedes-Rubio et al., 2010). Activation of the prenylated protein Rac1 has been shown to contribute to increased oxidative stress in AD (Lee et al., 2002; Chéret et al., 2008). Inhibition of Rho prenylation decreases total and phosphorylated tau levels (Hamano et al., 2012). We and others have also shown that manipulation of isoprenoid and protein prenylation levels modulates synaptic plasticity and cognitive function in animal models (Mans et al., 2010, 2012; Li et al., 2006; Ye and Carew 2010; Costa et al., 2002). Intriguingly, inhibiting the level of FPP/farnesylation, but not GGPP/geranylgeranylation, enhances hippocampal synaptic

plasticity in brain slices of mature C57BL/6 mice (Mans et al., 2012; Parent et al., 2014). Consistent with these results, our most recent study indicates that haplodeficiency in farnesyl transferase, but not geranylgeranyl transferase-1, rescues cognitive function as well as attenuates Aβ-associated neuropathology and neuroinflammation in a mouse model of AD (Cheng et al., 2013a). Taken together, these findings strongly suggest that alteration of small GTPases is implicated in the pathogenesis of AD and that modulation of protein prenylation, in particular protein farnesylation, may present a potential therapeutic strategy for AD.

CHAPTER 2 – TRANSGENIC EXPRESSION OF CHOLESTERYL ESTER TRANSPORT PROTEIN DOES NOT AFFECT AMYLOID PATHOLOGY IN APP/PS1 MICE

Introduction:

AD is the most common form of age-related dementia (Alzheimer's Association 2017). AD is clinically diagnosed by progressive memory loss and biochemically characterized by amyloid deposits and neurofibrillary tangles. The importance of lipoprotein metabolism has been well established in the development and progression of AD (Vitali et al., 2014). In fact, apoE is the strongest genetic risk factor in the development of AD (Corder et al., 1993). Brain-derived apoE is secreted from astrocytes (Linton et al., 1991) and forms particles similar to HDL particles in the plasma (Stukas et al., 2014).

Several types of apolipoproteins are not able to cross from the periphery into the CNS. ApoA-I is not produced in the brain (Dietschy and Turley 2001) and is minimally capable of crossing into the CNS, possibly via SR-BI mediated transcytosis (Rigotti et al., 1997) or choroid plexis (Stukas et al., 2014). Other important apolipoproteins detected in the CNS include apoJ, apoA-II, apoA-IV, apoD, and apoH (Thanopoulou et al., 2010). The general structure of lipoprotein particles includes a hydrophobic core surrounded by a hydrophilic shell. The hydrophobic core contains neutral, non-polar lipids, predominantly TG and CE. The hydrophilic shell consists of primarily PL, unesterified FC, and various apos, which mediate interactions with a variety of other molecules including enzymes, transporters, and receptors through a dynamic process.

HDL, often known as the “good cholesterol”, is widely known to be cardio-protective (Barter et al., 2004) and emerging evidence to the cognitive benefits of HDL are currently being described (Hottman et al., 2014; Lewis et al., 2010). HDL-like particles in the CSF are formed from brain-derived apoE primarily secreted by astrocytes and microglia. Newly transcribed and secreted apoE forms discoidal particles that are lipidated though several enzymatic steps to form mature, spherical HDL-like particles. One major protein responsible for the lipidation of apoE containing particles is the ABCA1. ABCA1 interacts with poorly lipidated apoE on the surface of astrocytes to form HDL-like particles (Hirsch-Reinshagen et al., 2004; Wahrle et al., 2004). This process is known as RCT and is important in cholesterol homeostasis in the brain and periphery.

The brain contains roughly 25% of the body’s total cholesterol (Dietschy and Turley 2004). Cholesterol is unable to cross the BBB. Therefore, all cholesterol found in the brain is synthesized by astrocytes and oligodendrocytes and is isolated from peripheral cholesterol reserves. Excess cholesterol in the brain can be metabolized by cytochromes P450 (cyp) 46A1 to form 24s-hydroxycholesterol which can be effluxed through the BBB into the periphery (Dietschy and Turley 2001; Panzenboeck 2002).

LCAT is a major enzyme responsible for the production of cholesteryl and oxysterol esters in plasma (Rousset et al., 2011). LCAT is primarily produced peripherally in the liver but is also produced in a smaller amount by astrocytes in the CNS (Hirsch-Reinshagen et al., 2009). Recent literature reports that the genetic deletion of LCAT from mice does not impair amyloid metabolism in APP/PS1 mice (Stukas et al., 2014).

CETP also plays an important role in lipoprotein remodeling in the plasma and CNS. CETP functions to convert HDL to other, lower density, lipoproteins – effectively lowering protective levels of HDL and aiding in the formation of lower density fractions that have been linked to several cardiac and neurological diseases and complications (Barkowski and Frishman 2008). CETP has been isolated from human CSF, which is thought to have a similar function in the CNS as in the periphery. Interestingly, mice lack a CETP gene. This results in a preservation of the HDL fraction since endogenous CETP in humans facilitates the transport of cholesteryl esters and triglycerides between lipoproteins. Our previous research has shown that elevated HDL cholesterol levels improve cognitive learning and memory in an AD mouse model (Lewis et al., 2010). Conversely, genetically reduced HDL cholesterol levels drastically decreased cognitive performance in mice (Lefterov et al., 2010).

The role of CETP in AD is not known. To address the question of whether CETP expression affects Aβ levels, we crossed CETP-expressing transgenic mice with the APP/PS1 model of AD and generated the triple transgenic APP/PS1/CETP mice. We found that the presence of CETP reduced the level of HDL-C but did not significantly affect the soluble and aggregated Aβ levels or plaque load in the cortex and hippocampus in the APP/PS1 mice at 6 and 9 mo. Consistently, expression of CETP did not exacerbate cognitive deficits in APP/PS1 mice. These results suggest that CETP expression itself or CETP-induced lowering of HDL-C is not sufficient to influence β-amyloidogenesis and cognitive function in APP/PS1 mice at young ages (6-9 mo).

Materials and methods:

Animals:

APPPS1 double transgenic mice (B6C3-Tg (APPswe, PSEN1dE9) 85Dbo/J; stock number 004462 (Jankowsky et al., 2004)) on a mixed genetic background (B6xC3H) were crossed with mice expressing CETP under its endogenous promotor ((C57BL/6J x CBA/J)F1; backcrossed to C57BL/6 for at least ten generations; stock number 003904 (Jiang et al., 1992); The Jackson Laboratory (Bar Harbor, ME)). Littermate controls were used for all genotype comparisons. All transgenes are kept heterozygotic. Genotypes of the mice were determined by PCR analysis of genomic DNA from tail biopsies with gene-specific primers. All animal procedures used for this study were prospectively reviewed and approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Blood collection and brain tissue preparation:

The mice were deeply anesthetized using Ketamine/Xylazine. Blood was collected using heparin rinsed syringes via from cardiac puncture. Following perfusion with ice-cold PBS, brains were cut sagittally into left and right hemispheres. The left hemisphere was fixed in 4% paraformaldehyde for histological analysis. Cortical and hippocampal regions were separated from the right hemisphere and snap-frozen in liquid nitrogen and stored at -80 °C for biochemical analysis.

Cholesterol and paraoxonase analysis:

Fresh plasma was separated from whole blood via centrifugation at 3k rpm for 10 min at 4°C. Total plasma was treated with HDL cholesterol reagent (Pontine Scientific) to separate HDL-cholesterol from non-HDL-cholesterol. Total cholesterol and HDL-cholesterol fractions were then measured by the Infinity™ Cholesterol Liquid Stable Reagent (Thermo Scientific, Middletown, VA) as described previously (Cheng et al., 2013b; Cao et al., 2007; Li et al., 2006; Cao et al., 2006).

Brain amyloid β enzyme-linked immunosorbent assay:

Brain homogenates were prepared as we described previously (Cao et al., 2007; Lewis et al., 2010). Commercial ELISA kits (Invitrogen) were used to measure Aβ40 and Aβ42 levels in carbonate-soluble and -insoluble (guanidine-soluble) fractions according to the manufacturer's protocol.

Immunohistochemical analysis and quantification of amyloid β deposition:

Protocols for immunohistochemical (IHC) analysis have been previously described (Cao et al., 2007; Billings et al., 2005; Lewis et al., 2010). Briefly, 4% paraformaldehyde fixed tissues were sectioned at 50 µm using a vibratome (Leica Microsystems Inc). Tissue sections were stored in PBS with 0.01% sodium azide at 4 °C. Tissue sections were subjected to free-floating immunostaining using the ABC kit (Vector Laboratories, Burlingame, CA) to detect Aβ, activated microglia, and activated astrocytes. The primary antibody 6E10 (signet) was used for assessing Aβ deposition, IBA-1 antibody (Wako) for

determining activated microglia, and GFAP antibody (Millipore) for assessing activated astrocytes. Immunoreactivity of Aβ, IBA-1, and GFAP in the cortex and hippocampus of mouse brains were quantified using a histomorphometry system (Image-Pro Plus, MediaCybernetics, Rockville, MD).

Behavioral assessment:

Several AD-related behavioral functions were assessed in the mice. The testing schedule included open field (days 1-3), the elevated plus-maze for anxiety levels (days 4-5), and spatial learning in the Morris water maze (days 6-11). All equipment and software were purchased from SD Instruments (San Diego). All testing procedures have been previously described (Li et al., 2006; Cao et al., 2007; Lewis et al., 2010; Cheng et al., 2013b).

Briefly, the Morris water maze tests the special learning and memory systems of a mouse. A round basin is filled with water and surrounded with extra-maze visual cues for orientation. The acquisition of the spatial task consists of placing the mice next to and facing the wall successively in the north, east, south, and west quadrants. An escape platform is hidden 0.5cm below the water level in the middle of the northeast quadrant. In each trial, the mouse is allowed to swim until it finds the hidden platform, or until 60 s have elapsed, whichever occurs first. If the mouse failed to find the hidden platform within 60 s, the mouse was guided to the platform. The mice remained on the platform for 15 s before being partially dried and returned to their home cage. The escape latency and swim path were recorded by the ANYMAZE system (San Diego Instruments, San Diego) for

four trials daily over 5 days. The probe trial was performed on the 6th day of behavioral assessments. The probe trial was conducted by removing the platform and placing the mouse in the north quadrant as previously described. The time spent in the previously correct (target) quadrant and previous platform location crossovers were measured in a single 60 s trial. Following the probe trial, the visible acuity of the mice was tested. The escape platform was lifted to 1cm above the water level and shifted to the southwestern quadrant. A visible marker was added to the escape platform as a viewing aid. This visual trial was used to evaluate the visual acuity of the mice.

Statistical analysis:

Data are expressed as means ± S.E. Comparison of different genotype groups was performed by Student's *t* test and repeated measures of analysis of variance. $p < 0.05$ was considered statistically significant.

Results:

CETP transgenic expression has no negative effects during development or postnatal body weight gain:

Our breeding strategy crossed APP/PS1 mice with CETP mice to produce four genotypes: Wild Type (WT), CETP, APP/PS1, APP/PS1/CETP. Each genotype has an expected occurrence of 25% of total mice. Our breeding strategy did not yield a genotype distribution that was significantly different from expected ($\chi 2 = 0.82$; $p = 0.84$) (**Fig. 2.1**). These data provide evidence that there are no negative developmental consequences in

mice expressing CETP, which ensures that any differences seen between CETP expressing and non-CETP expressing mice are not linked to developmental differences. We also did not observe any decrease in body weight due to CETP expression (**Fig. 2.2)**. This is an indication that the health of the mice is similar across genotypes.

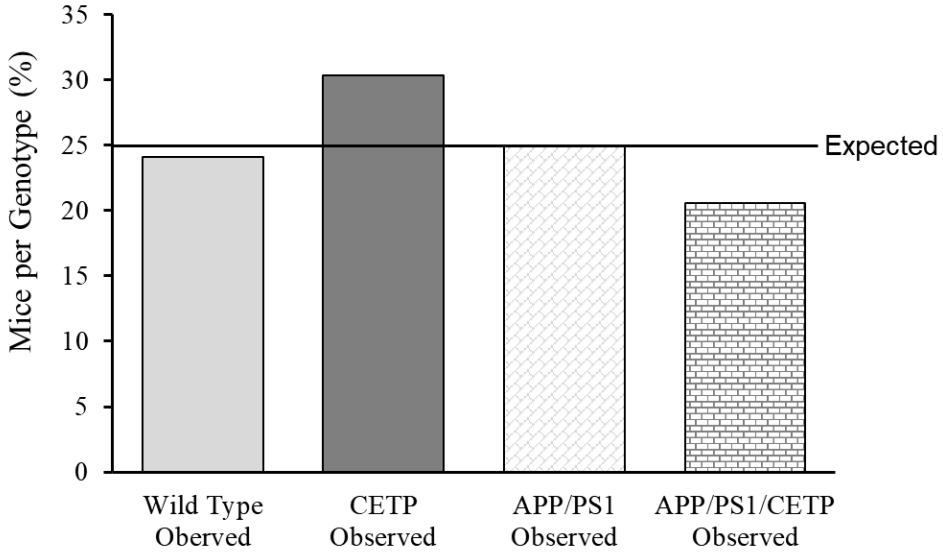

Fig. 2.1 – CETP tg expression does not alter the survival rate of mice. Genotype was determined from tail biopsy via PCR with genotype specific primers. n = 23-34 mice per group.

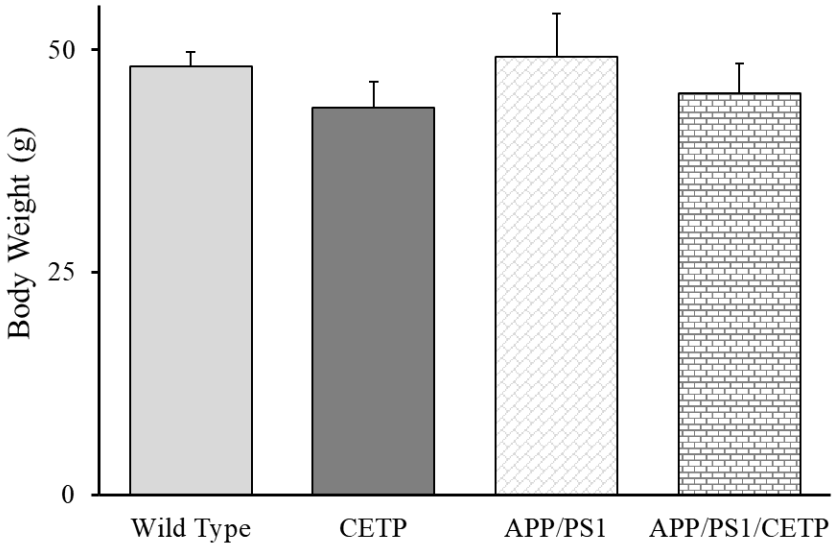

Fig. 2.2 – CETP tg expression did not perturb body weight. Body weight data was collected from 9 mo mice prior to dissection. n = 9-15 mice per group.

CETP expression leads to reduced levels of total cholesterol and HDL cholesterol:

We analyzed the plasma total and HDL cholesterol levels in the four genotypes. As hypothesized, CETP tg expression in CETP and APP/PS1/CETP mice decreased the total plasma cholesterol level by roughly 30% compared to non-CETP expressing mice (**Fig. 2.3a**). These results provide evidence that human CETP expressing in a mouse is still enzymatically active on mouse lipoproteins. TC is reduced because mice carry a majority of their FC in the HDL fraction (Yin et al., 2012) and CETP activity has a net transfer from HDL to lower density lipoprotein fractions. This characterization of CETP expressing mice helps establish them as a more humanized mouse model for future cholesterol research. Interestingly, APPPS1 had 20% higher TC than WT.

Among human patients with AD, there exists a strong inverse correlation between HDL-cholesterol levels and cognitive impairment. Therefore, in our next experiment we isolated the HDL-cholesterol fraction from the non-HDL-cholesterol to better understand the role HDL plays in AD. Similar to the total plasma cholesterol changes we detected, CETP expressing in CETP and APP/PS1/CETP mice caused a decrease in HDL-C levels by ~ 25% compared to non-CETP expressing littermate controls (**Fig. 2.3b**). Again, similar to the TC analysis, APP/PS1 mice had a 20% higher HDL-cholesterol concentration than WT. The HDL-C/TC plasma ratio did no change (**Fig. 2.3c**) One plausible explanation for the increase in TC and HDL-C could be an upregulation of RCT proteins and other HDL precursors to combat AD progression in these mice.

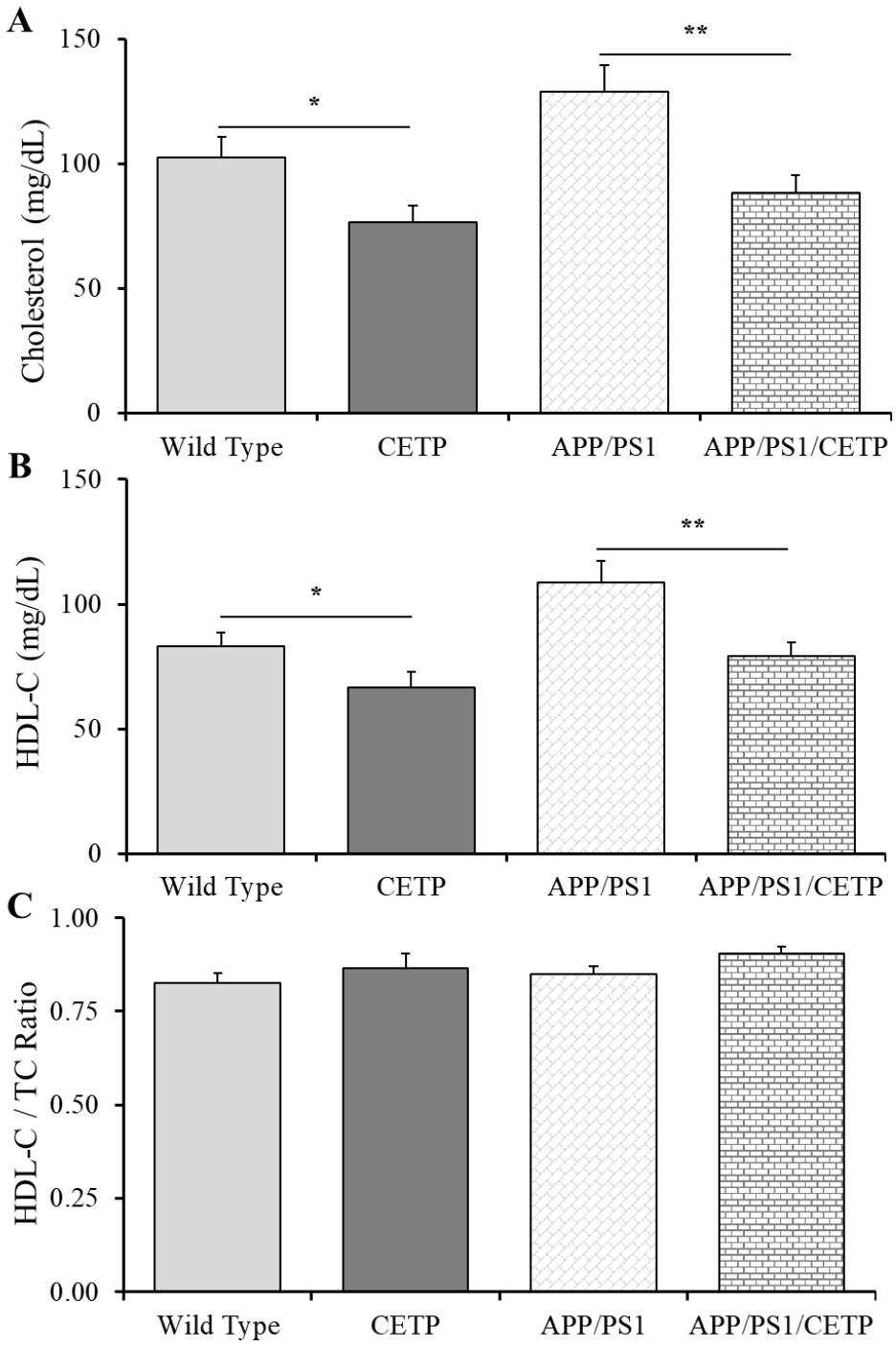

Fig. 2.3 – Transgenic expression of CETP significantly reduces total cholesterol and high-density lipoprotein cholesterol levels. A) Total cholesterol and **B)** HDL-C quantification from plasma of dissected 9 mo mice. **C)** HDL-C/Total Cholesterol ratio is unchanged. n=12 mice per genotype (6 male and 6 female). * $p < 0.05$. ** $p < 0.01$.

Expression of CETP did not affect HDL-associated paraoxonase 1 activity:

One major protective effect of HDL is in its anti-atherosclerotic and anti-inflammation functions. These functions are partially performed though the enzymatic breakdown of oxidized-lipids via paraoxonase 1 (PON1) activity. To study one aspect of HDL's anti-inflammation functions in AD, we determined the enzymatic activity of PON1 as a measure of the plasmas anti-inflammation capability. Lower PON1 activity results in reduced detoxification, and therefore, is presumed to be less capable of protecting against inflammation. The results showed that all genotypes had similar PON1 activity (**Fig. 2.4**), indicating that there is no reduction in the anti-inflammation properties of HDL in CETP expressing mice compared to their non-CETP expressing littermate controls. PON1 is associated with HDL particles and thus a decrease in HDL was expected to have a decrease in the PON1 enzymatic activity (Garin et al., 2006). However, it is possible that the PON1 assay was insufficiently sensitive to detect a decrease in PON1 activity or there was a compensatory increase of PON1 activity with reduced HDL levels in CETP-expressing mice.

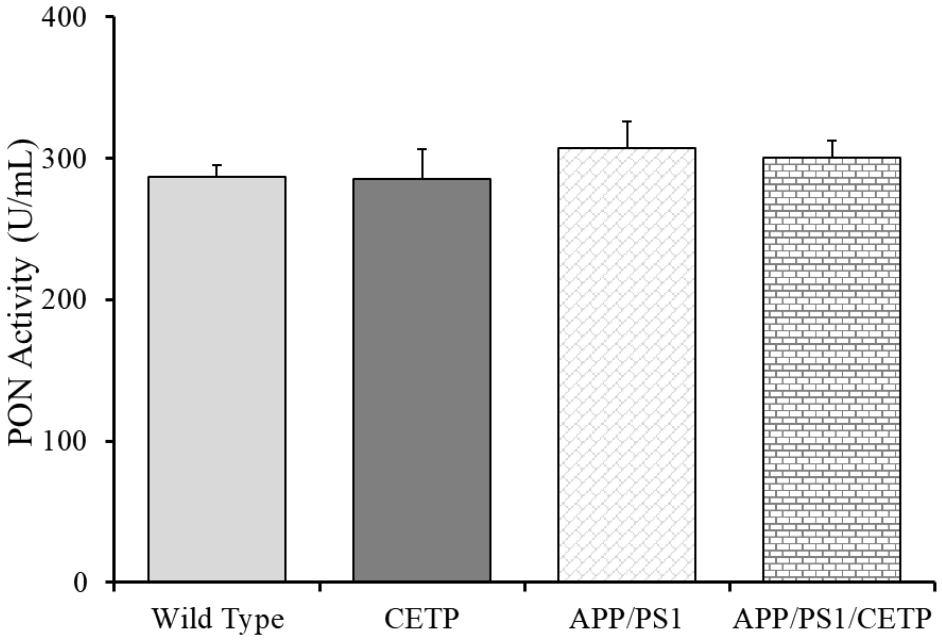

Fig. 2.4 – Transgenic expression of CETP does not reduce high-density lipoprotein associated paraoxonase 1 activity. PON1 enzymatic activity from HDL fraction quantification of 9 mo mice. n = 12 mice per genotype (6 male and 6 female).

CETP expression has no effect on Aβ levels and deposition in APP/PS1 mice:

Previous literature demonstrates the importance of HDL-cholesterol concentration on amyloidogenesis in mouse models of AD. Low HDL-cholesterol accelerates Aβ plaque formation and elevated HDL-cholesterol slows disease progression. We observed a 25% decrease in HDL-cholesterol in our CETP expressing mice. To quantify the effects of the 25% reduction in HDL-cholesterol on Aβ levels in these mice, hippocampus and cortical brain tissue samples were prepared from 6 mo and 9 mo APP/PS1 and APP/PS1/CETP mice. These samples were analyzed by Aβ40 and Aβ42 ELISA kits to quantify the concentrations of Aβ in each brain region. Normally, these APP/PS1 transgenic mice start to form plaque pathology around 5-6 mo. Therefore, we quantified Aβ concentrations in 6 mo mice to determine if there was an accelerated onset of plaque pathology in mice with

low HDL-cholesterol (**Fig. 2.5**). Interestingly, there was no statistical difference between APP/PS1 and APP/PS1/CETP in either cortical or hippocampal brain regions. These data suggest that the decrease in HDL-cholesterol induced by the CETP transgene in mice was insufficient to accelerate Aβ level accumulation.

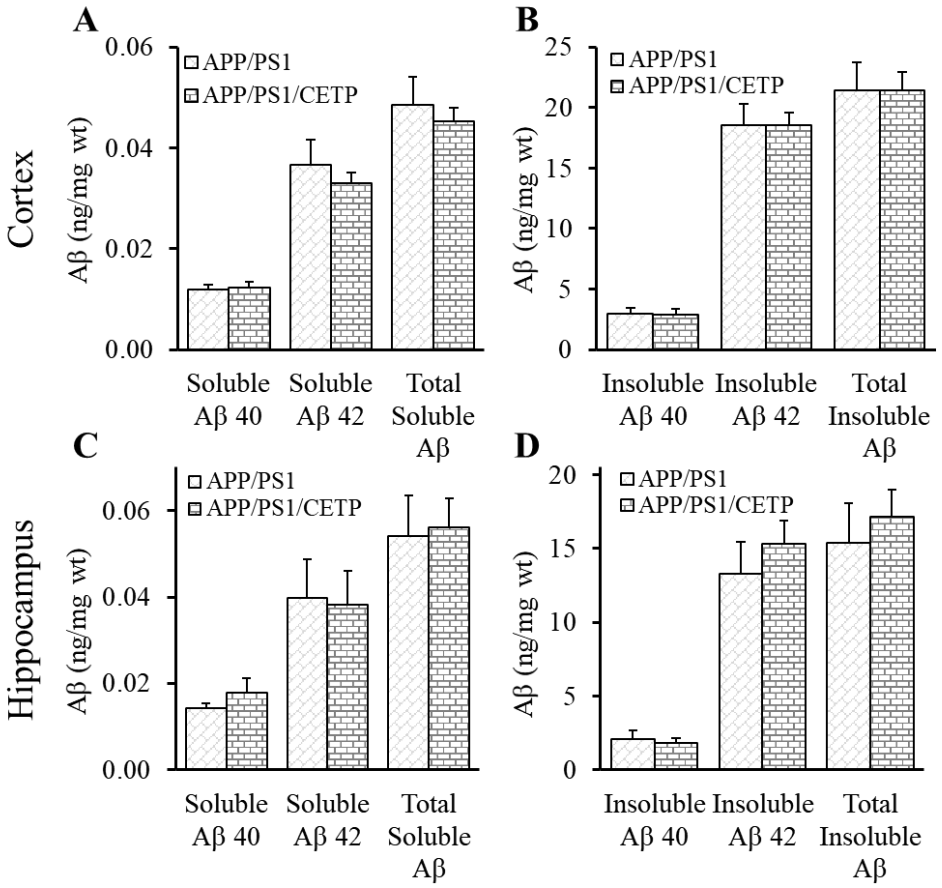

Fig. 2.5 – Transgenic expression of CETP does not change Aβ level in cortex or hippocampus of 6 month old mice A) Carbonate soluble and **B) insoluble (**guanidine soluble) amyloid in cortex. **C)** Carbonate soluble and **D) insoluble (**guanidine soluble) amyloid in hippocampus. n = 8 per group.

Next, we studied 9 mo cohort of APP/PS1/CETP and APP/PS1 mice to determine if there was any changes in disease progression with chronically reduced HDL-cholesterol (**Fig. 2.6**). Consistent with the 6 mo cohort, there was no statistical difference between APP/PS1 and APP/PS1/CETP in either cortical or hippocampal brain regions.

Interestingly, we observed a ~15% increase in insoluble Aβ 42 in APPPS1/CETP mice compared to APPPS1 littermate controls. Although this increase was not statistically significant, the trend of increase in soluble Aβ in older APP/PS1/CETP mice suggests that the effects of sustained decrease in HDL-cholesterol levels may be manifested during aging.

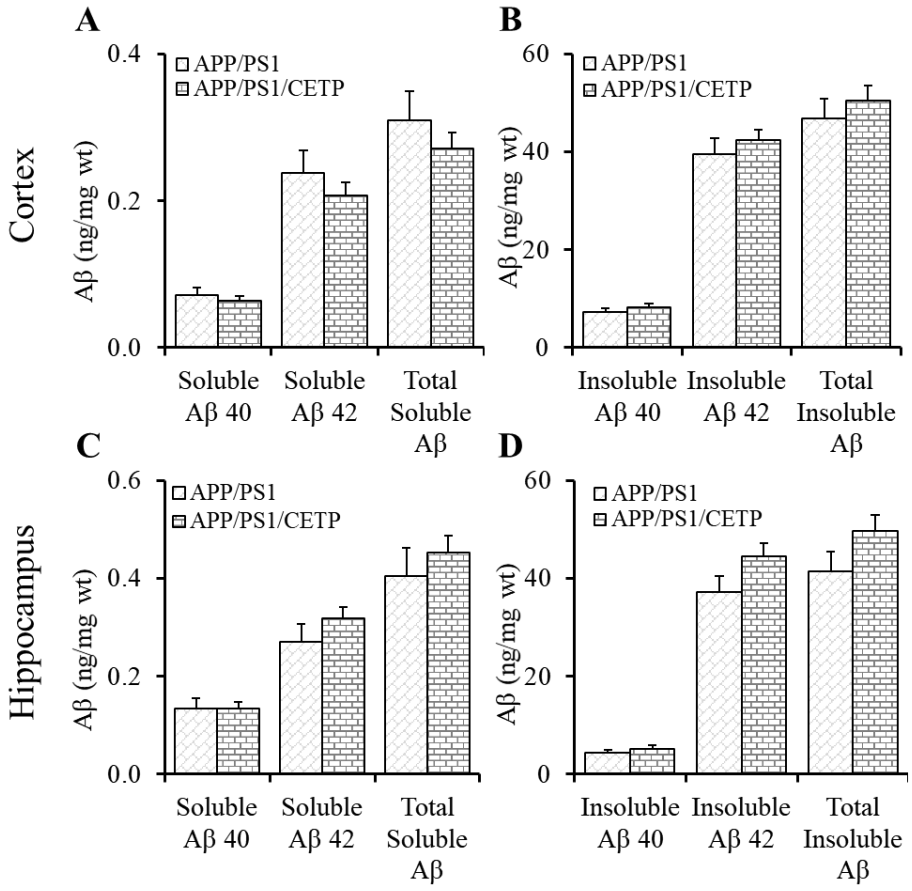

Fig. 2.6 – Transgenic expression of CETP does not change Aβ level in cortex or hippocampus of 9 month old mice. A) Carbonate soluble and **B) insoluble (**guanidine soluble) amyloid in cortex. **C)** Carbonate soluble and **D) insoluble (**guanidine soluble) amyloid in hippocampus. n = 10 per group.

To determine the effects of CETP expression on Aβ pathology and neuroinflammation further, we quantified Aβ plaque deposition in APP/PS1/CETP versus

APP/PS1 mice by immunohistochemical analyses. All IHC results were collected from our 9 mo cohort to better elucidate the chronic effects reduced HDL-cholesterol has on AD pathogenesis. We saw no significant difference in Aβ plaque IHC staining in the cortex or hippocampus between APPPS1 and APPPS1/CETP (**Fig. 2.7a,b**). These results are consistent with the ELISA data described in Fig. 2.6.

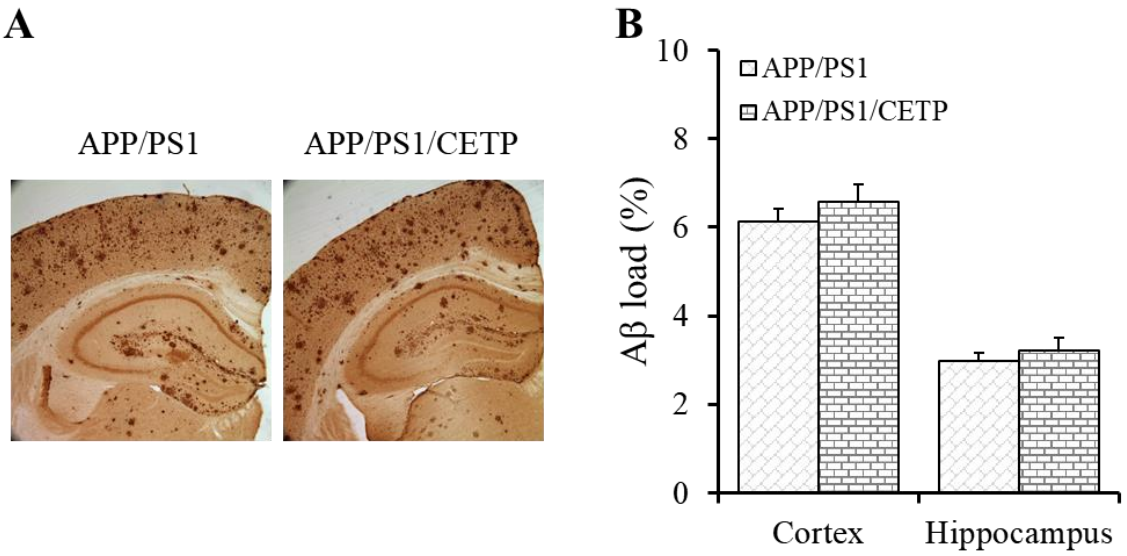

Fig. 2.7 – CETP tg expression does not affect plaque deposition in 9 month old APP/PS1 mice. A) Representative images **B)** Quantification of immunohistochemical analysis comparing APP/PS1 and APP/PS1/CETP brain sections. n = 10 per group.

CETP expression does not influence neuroinflammation:

Astrocytes and microglia are important regulators of inflammation and neuronal health in the CNS. Astrocytes are known to upregulate glial fibrillary acidic protein (GFAP) in response to inflammatory stimuli (Oblinger and Singh 1993), such as Aβ deposition (Jantaratnotai et al., 2003; Damjanac et al., 2007). To determine the extent of astrocyte activation in response to Aβ deposition, we quantified the immunohistochemical staining of GFAP in our 9 mo cohort. There was no change between APP/PS1/CETP and

APP/PS1 littermate controls (**Fig. 2.8**). These results indicate that transgenic expression of CETP had no effect on the response of astrocytes to Aβ deposition. Both genotypes had on average ~2.5% of their total brain area stained positive for GFAP, indicating similar levels of activated astrocytes. To determine the response of microglia to Aβ deposition, we quantified IHC staining with IBA1. Consistently, there were no significant differences between CETP tg mice and their non-tg littermate controls (**Fig. 2.8**). These results indicate that CETP-induced changes in the cholesterol profile were not sufficient to influence the status of neuroinflammation.

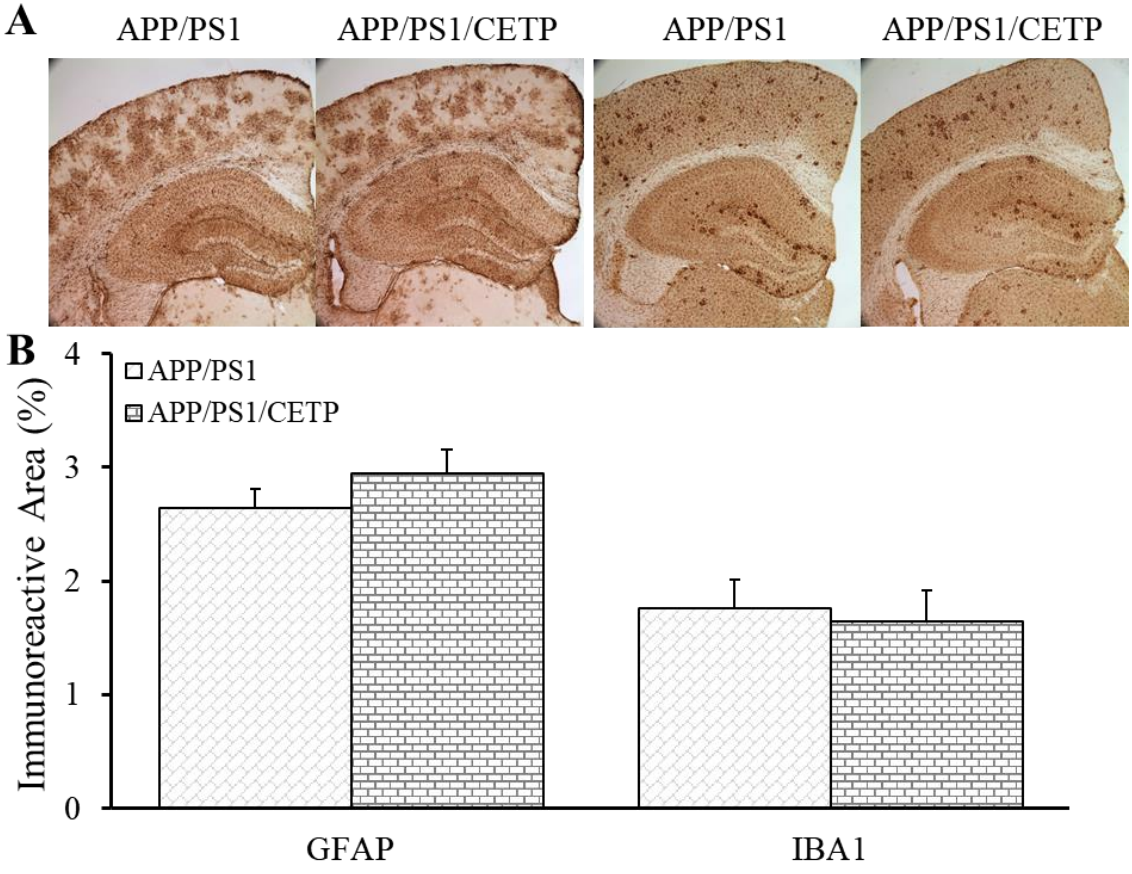

Fig. 2.8 – CETP tg expression does not affect neuroinflammation markers in 9 month old APP/PS1 mice. A) Representative images **B)** Quantification of immunohistochemical analysis comparing APP/PS1 and APP/PS1/CETP brain sections. n = 6 per group.

Cognitive decline is not exacerbated with cholesteryl ester transfer protein transgenic expression:

We performed a series of behavioral assessments to determine whether CETP activity affects behavioral function including learning and memory compared to APP/PS1 mice. The open field test was performed to determine if any differences in general locomotor activity levels or anxiety level in our mice existed. We did not observe any difference between the genotypes in the distance traveled (locomotor activity) with an average of 35.5 ± 2.0 meters traveled on day one, down to 25.0 ± 1.6 meters on day three of testing (**Table 2.1)** (n = 14-20 mice per genotype) or time spent in the center zone (anxiety) with an average of 9.6 ± 0.8s on day one, down to 5.2 ± 0.7s on day three of testing **(Table 2.1)**. All genotypes had normal habituation patterns, exploring the open field space less with each successive trial.

Table 2.1 – Locomotor activities of experimental mice in the open field test

	Day 1		Day 2		Day 3	
	Distance (m)	Time in Center (s)	Distance (m)	Time in Center (s)	Distance (m)	Time in Center (s)
Wild Type	34.7 ± 3.0	10.8 ± 1.7	28.9 ± 2.7	8.6 ± 1.7	26.8 ± 3.3	6.6 ± 1.6
CETP	31.5 ± 2.7	11.2 ± 2.0	29.4 ± 2.2	8.2 ± 0.9	23.9 ± 2.5	5.1 ± 1.2
APP/PS1	37.4 ± 6.0	7.2 ± 1.3	27.9 ± 4.1	4.0 ± 0.9	22.8 ± 3.5	3.5 ± 1.3
APP/PS1/CETP	39.4 ± 5.0	8.4 ± 1.6	32.7 ± 4.4	7.6 ± 1.6	26.1 ± 4.0	5.0 ± 1.3

Next, we performed the elevated plus maze test to measure anxiety. Mice that are less anxious will spend a larger proportion of their time in the open arms of the maze. We saw no differences between any genotypes in the elevated plus maze indicating equal anxiety levels among groups. The average time spent on the open arms was 50 ± 4.5s on day 1, down to 22.8 ± 2.6s on day three of testing **(Table 2.2)** (n = 14-20 mice per genotype). Similarly, all genotypes displayed normal habituation patterns after exploring the novel environment.

Table 2.2 – Anxiety levels of experimental mice in the elevated plus maze test

	Day 1	Day 2
	Time in open arm (s)	Time in open arm (s)
Wild Type	48.2 ± 5.9	26.0 ± 3.8
CETP	52.0 ± 7.1	25.1 ± 4.8
APP/PS1	52.2 ± 11.6	20.8 ± 7.3
APP/PS1/CETP	47.9 ± 12.9	17.7 ± 5.9

The Morris water maze test was performed on the 9 mo cohort. As is expected, wild type mice readily learned to locate the hidden platform during the 5-day acquisition phase and APP/PS1 showed learning impairments (**Fig. 2.9a**). These two genotypes serve as control groups for the APP/PS1/CETP genotype mice. APP/PS1/CETP mice performed similarly to APP/PS1 mice indicating there was not a worsening of cognitive deficits with CETP expression. Next, to assess memory retention, the mice were subjected to a single probe trial 24 h later (**Fig. 2.9b**). WT and CETP mice had significantly more platform

crossovers than APP/PS1 and APP/PS1/CETP mice demonstrating memory deficits of mice with APP/PS1. Overall, these behavioral results demonstrate that the alterations to the cholesterol profiles elicited with CETP expression were not dramatic enough to exacerbate behavioral outcomes normally seen in APP/PS1 mice. Additionally, CETP expression alone, without APP/PS1 genotype, did not negatively impact the learning curves of mice compared their WT littermates.

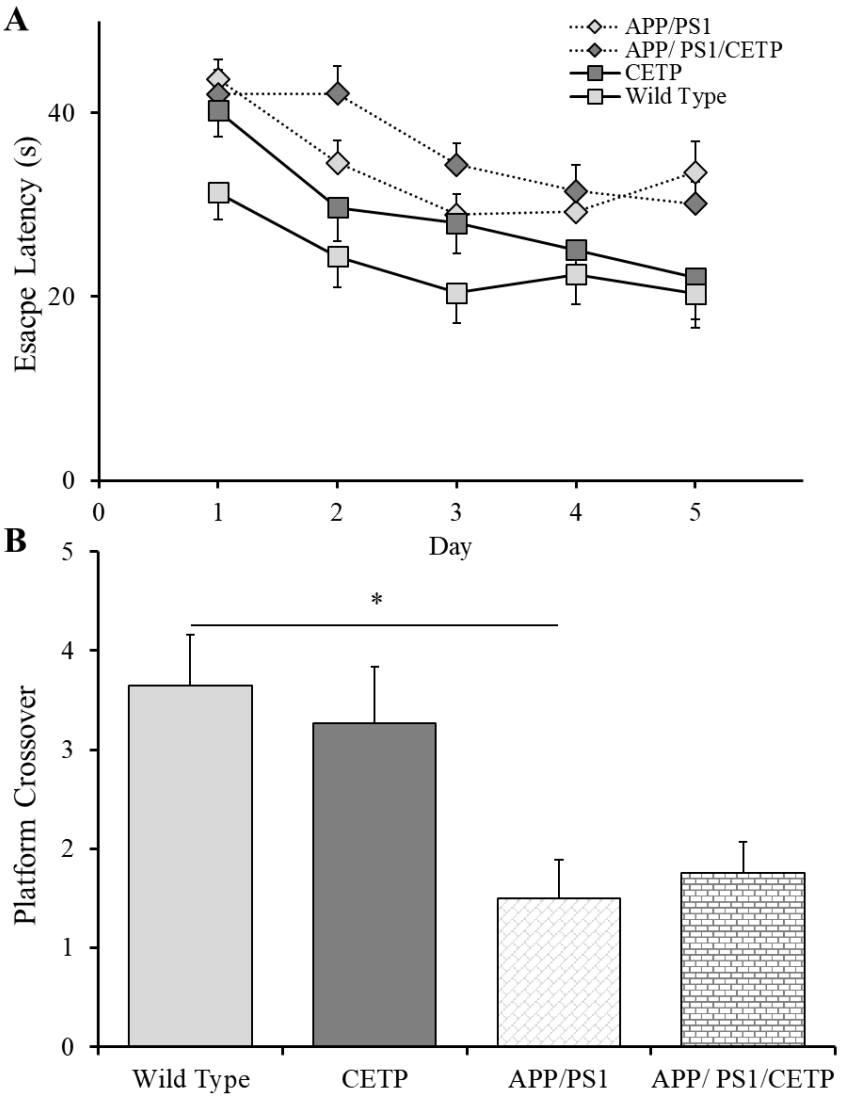

Fig. 2.9 – Mice expressing CETP perform similarly as their non-CETP expressing littermate controls. A) The acquisition phase and **B)** the retention phase of the Morris water maze. APP/PS1 mice showed learning and memory deficits compared to wild type controls. n = 14-20 per group.

Discussion:

In the plasma, HDL interacts with cells and other lipoprotein particles through multiple receptors, transporters, and enzymes (Hottman et al., 2014). Previous animal studies suggests that overexpression of human apoA-I increases HDL levels and promotes cognitive function (Lewis et al., 2010) whereas genetic deletion of apoA-I markedly decreases HDL levels and exacerbates cognitive deficits in APP/PS1 mice (Lefterov et al., 2010). In the present study, we found that the addition of CETP to a mouse genome alters cholesterol profiles of mice by reducing the HDL-C levels. However, this reduction is not as drastic as the change seen in apoA-I knockout mice, and therefore, this genetic change does not seem to be sufficient to elicit other pathological or behavioral changes in mice. Future directions could include working with hamster or other animal models with cholesterol profiles more similar to those of humans (i.e. higher LDL-C and lower HDL-C).

CETP expression caused a modest alteration to the cholesterol profile. It is possible that if CETP expression elicited a more robust conversion of protective HDL to other non-HDL cholesterol pools, the cognitive effects would be more apparent. Interestingly, there was a trending increase in insoluble Aβ levels in the 9 mo cohort. This trend might continue to widen with age to the point where the effect size would be significant at more advanced ages. It is also worth noting that soluble Aβ levels are an important marker for AD severity because they are generally regarded as more toxic than their insoluble plaque form (Larson & Lesné, 2012).

One important technical aspect to be cognizant of is the challenge in showing worse memory deficits when the learning ability is already impaired in APP/PS1 mice. The APP/PS1 mice at the age tested were already poor performers in the Morris water maze and found the target platform at near the rate of random chance; thus, worsening performance might require a more sensitive behavioral test such as the contextual fear conditioning or the Barnes maze test.

Future directions include examining additional biomarkers such as CAA, which could give more complete insight into the pathological changes that occur with CETP tg expression. CAA levels could correlate more strongly with behavioral outcomes, even when plaque pathology is unchanged (Lewis et al., 2010). Additionally, a more sensitive assay such as the Barnes maze could help detect smaller cognitive differences in mice.

Recently, there have been several CETP inhibitors tested in the cardiovascular field to increase HDL-cholesterol in patients. These class of drugs raised HDL by 44-133% depending on the particular compound and dose. However, these drugs have failed the development or clinical testing phase to date. The fact that dramatic increases in HDL do not confer the expected benefits demonstrates the amount of knowledge still to be attained in this field. This has led to recent discussions about the quantity vs quality of HDL (Tziomalos, 2016). In fact, a large scale clinical trial showed that HDL-C level and all-cause mortality did not correlate linearly, instead, the lowest and highest HDL-C patients showed higher all-cause mortality than the more average patients (Bowe et al., 2016). Unfortunately, an agreed upon gold-standard biomarker for HDL functionality remains

elusive. Therefore, using HDL-C as an imperfect substitute may continue until such a biomarker is discovered.

Overall, this research suggests an incomplete understanding of the interplay between role of CETP activity, HDL effects, and AD pathogenesis. More data will need to be collected to further elucidate the potential mechanisms involved between cholesterol metabolism and AD pathology.

CHAPTER 3 – TREATMENT WITH A CLUSTERIN/APOJ PEPTIDE REDUCES AMYLOID PATHOLOGY AND RESCUES MEMORY FUNCTION IN APP/PS1 MICE

Introduction:

AD is the most common progressive neurodegenerative disorder and poses an ever-growing challenge to health care institutions and economic systems worldwide. In the United States, more than five million people are currently suffering from AD and this number is expected to continue rising due to our aging population (Alzheimer's Association, 2017). Currently, there is no effective treatment available to slow or reverse AD. This fact emphasizes the need to develop new therapeutic strategies to prevent or delay AD.

A recent genome-wide association studies (GWAS) discovered that a single nucleotide polymorphism (SNP) within the gene of clusterin (*CLU*, also known as apolipoprotein J (apoJ)) is significantly associated with AD (Harold et al., 2009). The relationship between CLU polymorphisms and AD has been replicated by several independent research group (Carrasquillo et al., 2010; Corneveaux et al., 2010; Kamboha et al., 2012; Seshadri et al., 2010), making *CLU* one of the top-ranking genes associated with sporadic AD. ApoJ is a heterodimeric protein composed of α- and β-chains covalently linked by disulfide bonds (Murphy et al., 1988). Like apoE and other apolipoproteins, apoJ has multiple functions in the brain and periphery (Elliot et al., 2010). It is produced peripherally in the liver where it associates with HDL in the plasma, and in the brain it is

secreted primarily by glia, and is found in the CSF (Harr et al., 2002; Murphy et al., 1988; Pasinetti et al., 1994; Zwain et al., 1993; Saura et al., 2003). Interestingly, apoJ is more prominently expressed in the brain than in any other tissue (de Silva et al., 1990). ApoJ also serves as a molecular chaperon mediating cellular stress response under various disease conditions (Elliot et al., 2010). Multiple lines of evidence indicate that apoJ may be involved in the pathogenesis of AD. ApoJ has a high binding affinity for Aβ and is found in amyloid plaques (Ghiso et al., 1993; Giannakopoulos et al., 1998). The level of apoJ is associated with AD progression in key brain regions such as the entorhinal cortex and hippocampus (May et al., 1990). Recent human studies also show that plasma levels of apoJ increases in AD patients and correlates with the severity of disease (Thambisetty et al., 2010; Schrijvers 2011). *In vitro* and *in vivo* studies demonstrate that apoJ inhibits Aβ aggregation (Matsubara et al., 1996; Narayan et al., 2012), enhances phagocytosis of Aβ aggregates (Bartl et al., 2001), and facilitates the clearance of Aβ across the BBB (Bell et al., 2007) as well as modulates inflammatory and immune functions (Tschopp and French 1994). In addition, apoJ and apoE have been shown to work cooperatively to suppress Aβ levels and deposition in mouse models of AD (DeMattos et al., 2004). Taken together, previous findings strongly suggest that apoJ plays a protective role in the development of AD. Therefore, strategies that improve beneficial functions of apoJ could be effective in preventing and/or treating AD. One promising strategy is to use a small biologic, such as a peptide, to mimic the beneficial effects of full length apoJ, which is easier to synthesize and deliver.

In the search for a small peptide that mimics the function of apoJ, several sequences representing amphipathic helixes in the mature apoJ protein were synthesized (Navab et al., 2005). A leading peptide is the 10-amino acid chain corresponding to amino acid residues 113 to 122 in apoJ, called apoJ[113-122] peptide. This peptide has been found to effectively inhibit oxidized lipid-induced inflammatory reactions *in vitro*, strongly mimicking the effects of full length apoJ. To test the function of the peptide *in vivo*, D-apoJ[113-122] peptide synthesized from all D-amino acids were used for oral administration to animals to avoid degradation in the digestive system. In apoE-deficient mice, a single oral dose of D-apoJ[113-122] peptide (100 μg) was able to rescue the anti-inflammatory property of HDL in a few hours and promote cholesterol efflux from macrophages. In a 24 week follow-up study, atherosclerotic lesions were dramatically decreased in apoE-deficient mice treated with D-apoJ[113-122] peptide (50 μg/g diet) (Navab et al., 2005). In monkeys, oral D-apoJ[113-122] peptide treatment was found to reduce the levels of oxidized lipids and enhanced the anti-inflammatory property of HDL (Navab et al., 2005). A recent physicochemical study of D-apoJ[113-122] peptide further provides the structural basis for the anti-inflammatory and anti-atherogenic properties of this peptide (Mishra et al., 2011).

However, whether the beneficial properties of D-apoJ[113-122] peptide can be harnessed to treat AD had not been explored. In the current study, we tested the therapeutic potential of D-apoJ[113-122] peptide to improve neuropathology and behavioral function in the APP/PS1 mouse model of AD. We found that APP/PS1 mice treated with D-apoJ[113-122] peptide (20 mg/kg BW; daily intraperitoneal administration) for 3 mo had

a dramatically lower plaque burden at 9 mo compared to PBS treated littermates. The plasma levels of Aβ were elevated in D-apoJ[113-122] peptide treated mice, indicating an enhanced clearance of Aβ across the BBB. As a result, D-apoJ[113-122] peptide treated mice had significantly improved memory retention and lower CAA. Intriguingly, these beneficial outcomes were observed under the condition that D-apoJ[113-122] peptide itself had a limited penetration across the BBB in APP/PS1 mice. These findings suggest that the D-apoJ[113-122] peptide could potentially be an effective therapeutic agent for AD.

Materials and methods:

ApoJ[113-122] mimetic peptide:

The apoJ[113-122] mimetic peptide is a 10 amino acid molecule [Ac-L-V-G-R-Q-L-E-E-F-L-NH2], derived from the 113th-122nd amino acid sequence of the full length apoJ protein. D-apoJ[113-122] peptide was purchased from American Peptide Company [Product #: 332520. Lot # U01086A1] with all D-amino acids to reduce proteolytic degradation and improve half-life.

Animals and treatment regimen:

Breeders for APP/PS1 double transgenic mice (B6C3-Tg (APPswe, PSEN1dE9) 85Dbo/J; stock number 004462) (Jankowsky et al., 2001) were purchased from The Jackson Laboratory (Bar Harbor, ME) and crossed with B6C3F1 mice to produce all experimental mice. All genotypes were determined via PCR amplification of genomic DNA using gene-specific primers.

D-apoJ[113-122] was dissolved in sterile PBS to a stock concentration of 5mg/mL (~4mM), rocked at room temperature (RT) for 3-4h until fully dissolved, aliquoted, and stored at -20°C. On the day of the injections, frozen D-apoJ[113-122] peptide stock was thawed and warmed to RT before injection. Mice received a daily intraperitoneal (IP) injection of D-apoJ[113-122] peptide (20 mg/kg BW), with a 25 gauge needle to the lower right side of the abdomen, starting at 6 mo of age. Mice were weighed weekly and injection volume was adjusted to ensure a consistent dose. All animal procedures used for this study were prospectively reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota.

Blood collection and brain tissue preparation:

The mice were deeply anesthetized, and blood was collected by cardiac puncture with heparin coated needles. Next, the animal was perfused with ice-cold PB. Brains were removed and cut sagittally into left and right hemispheres. The left hemisphere was fixed in 4% paraformaldehyde and cut into 50 micron sections for histological analysis. The right hemisphere was further separated into cortical, hippocampal, and cerebellar regions then snap-frozen in liquid nitrogen and stored at -80°C for biochemical analysis.

Brain amyloid β enzyme-linked immunosorbent assay and immunoblot analysis:

Snap frozen brain samples were homogenized and processed as previously described (Lewis et al., 2010; Cao et al., 2007; Cheng et al., 2013a). Commercial ELISA kits (Life Technologies) were used to measure the soluble (carbonate-soluble) and

insoluble (guanidine-soluble) levels of Aβ40 and Aβ42 according to the manufacturer's protocol.

For immunoblot analysis, aliquots of brain homogenate were separated by SDS-PAGE and blotted to nitrocellulose membranes. The membranes were incubated with specific primary antibodies against apoJ (Cat# AF2747, R&D Systems, Minneapolis, MN), and APP (Cat# 512700, Invitrogen, Carlsbad, CA; recognizes both full-length APP and the c-terminal fragments of APP). Primary antibody incubation was followed by an HRP-conjugated secondary antibody incubation. Signal was detected by the ECL Plus Western Blotting System (GE Healthcare) and quantified by the ImageJ software. The blots were stripped and re-probed with anti-tubulin monoclonal antibody (Sigma) as a loading control.

Cerebral amyloid angiopathy and amyloid β deposition analysis:

Protocols for Congo red staining for cerebral amyloid angiopathy analysis have been described previously (Wilcock et al., 2006; Clement and Truong 2014). Briefly, fixed brain tissues were sectioned at 50 micron using a Vibratome (Leica Microsystems Inc). Tissue sections were stored at 4 °C in PBS with 0.01% sodium azide and incubated with 0.2% Congo red solution for 30 minutes at RT. Excess Congo red stain was washed away with ethanol and sections were cover-slipped using the Permount mounting medium. The vessels were examined with a Nikon 55i Microscope with Transmitted Brightfield using a TRITC Cube (Excitation 528-553 Dichroic Mirror 565 Emission 590-650) and analyzed using Image-Pro Plus (MediaCybernetics, Rockville, MD).

The X-34 protocol for quantifying fibrillar amyloid deposits had been previously described (Styren et al., 2000). Tissue sections were stored at 4°C in PBS with 0.01% sodium azide and incubated with 10μM X-34 at RT. Excess X-34 was washed away with ethanol and sections were cover-slipped using permount. The vessels were examined with a Nikon 55i Microscope with Transmitted Brightfield using a DAPI Cube (Excitation 340-380 Dichroic Mirror 400 Emission 435-485) and analyzed using Image-Pro Plus (MediaCybernetics, Rockville, MD).

Behavioral assessment:

Several AD-related behavioral functions were assessed to determine any rescue effects of D-apoJ[113-122] peptide. The testing schedule included the open field (days 1–3) for habituation, the elevated plus-maze (days 4 and 5) for anxiety levels, and spatial learning in the Morris water maze (days 6–11). All equipment and software were purchased from SD Instruments (San Diego). All testing procedures have been described in detail previously (Li et al., 2006; Cao et al., 2007; Lewis et al., 2010).

Briefly, the Morris water maze consists of a round basin filled with water in a room containing extra-maze visual cues for orientation. The acquisition phase consists of placing the mice next to the wall rotating between the north, east, south, and west positions. The escape platform was hidden 1cm below the water level. In each trial, the mouse was allowed to swim until it successfully found the hidden platform or until 60 seconds (s) had elapsed. The escape latency and swim path length were recorded by the ANYMAZE System (San Diego Instruments, San Diego), with four trials each day for five days. On the

sixth day of the Morris water maze, a probe trial was conducted by removing the platform and placing the mouse next the north wall. The time spent in the previously correct (target) quadrant was measured in a single 60-s trial. Each mouse's visual acuity was assessed using a similar protocol with a visible escape platform lifted 1 cm above water level.

Determination of D-apoJ[113-122] peptide concentration in plasma and brain:

Frozen brain and plasma tissue samples were collected 0, 1, 2, 4, and 6 hours after IP D-apoJ [113-122] peptide injection and shipped overnight on dry ice to Absorption Systems. The D-apoJ[113-122] peptide concentrations in the plasma and brain were determined by a capillary LC-MS/MS method established at Absorption Systems.

Statistical analysis:

Data are expressed as means ± standard error (SE). Comparison of different treatment/genotype groups was performed by Student's *t* test and repeated measures of analysis of variance where appropriate. $p < 0.05$ was considered statistically significant.

Results:

Treatment with D-apoJ[113-122] peptide significantly reduced Aβ levels in the brain of APP/PS1 mice:

To determine the effect of D-apoJ[113-122] peptide treatment on the levels of Aβ in APP/PS1 mice, brain cortical tissue homogenate was prepared for the measurements of Aβ40 and Aβ42 levels in carbonate soluble and guanidine soluble (insoluble) fractions

using commercial Aβ ELISA kits. The results showed that the levels of insoluble Aβ40 and Aβ42 in the brain of D-apoJ[113-122] peptide treated mice were significantly lower compared to those of PBS-treated mice (**Fig. 3.1**). These results demonstrated that peripherally administered D-apoJ[113-122] peptide reduced the levels of aggregated Aβ in the brain. Interestingly, the level of soluble Aβ was not affected by the D-apoJ[113-122] peptide treatment, suggesting that the small soluble pool of Aβ maintains at an equilibrium level in the brain of APP/PS1 mice. Importantly, the total level of Aβ was robustly decreased in the brain of APP/PS1 mice treated with D-apoJ[113-122] peptide.

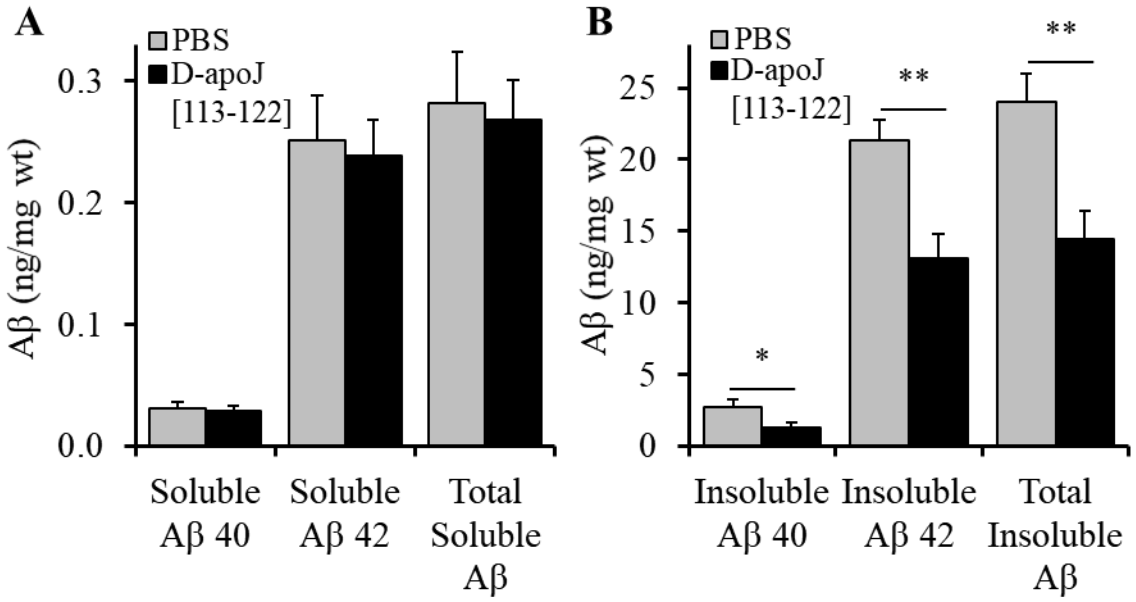

Fig. 3.1 – D-apoJ[113-122] peptide treatment reduces brain Aβ levels in APP/PS1 mice. The levels of $A\beta_{40}$ and $A\beta_{42}$ in **A)** carbonate and **B)** guanidine soluble fractions of cerebral cortical samples from APP/PS1 mice (n=9/group) were determined by ELISA. * $p < 0.05$; ** $p < 0.01$

Treatment with D-apoJ[113-122] peptide significantly reduced x-34 positive amyloid plaques in APP/PS1 mice:

To further determine the effect of D-apoJ[113-122] peptide treatment on amyloid deposition in the brain, we conducted histochemical analysis with the dye X-34. X-34 is a lipophilic and fluorescent derivative of Congo red. X-34 is a histochemical stain that can detect pathological accumulation of fibrillary forms of amyloid in Alzheimer's disease (Styren et al., 2000). Similarly, in APP/PS1 mice, X-34 strongly stains amyloid plaques. We compared staining intensities between PBS and D-apoJ[113-122] peptide treated mice and detected a robust 42% decrease in X-34 stained area (**Fig. 3.2**). These data further demonstrate that insoluble Aβ levels are reduced by chronic treatment with the D-apoJ[113-122] peptide.

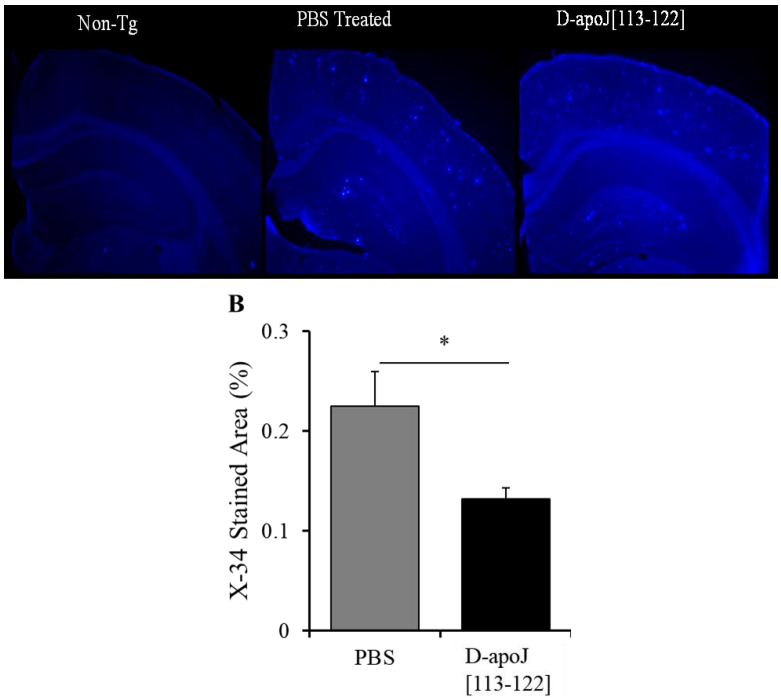

Fig. 3.2 – D-apoJ[113-122] peptide treatment reduces plaque pathology in APP/PS1 mice. A) Representative images of X-34 stained section. **B)** Quantification of the percent of the total cortical area stained by X-34, representing fibular Aβ (n=9/group). * $p < 0.05$

Treatment with D-apoJ[113-122] peptide significantly reduced CAA in APP/PS1 mice:

Impaired clearance of Aβ from the brain is a key pathological driver of AD (Mawuenyega et al., 2010). CAA is defined by the deposition of Aβ in cerebrovascular walls, which impairs the function of cerebral vessels and impedes Aβ clearance, resulting in accumulation within the brain. APP/PS1 mice develop CAA as well as parenchymal Aβ deposition in the brain. To examine the effect of D-apoJ[113-122] peptide treatment on CAA in APP/PS1 mice, we conducted histochemical analysis with the dye Congo red. Congo red is a histochemical stain that can detect fibrillary amyloid deposition in the vasculature as well as in the parenchyma of the brain. In APP/PS1 mice, Congo red strongly stains amyloid in cortical vasculature. We compared vascular staining intensities between PBS and D-apoJ[113-122] peptide treated mice and detected a dramatic 66.2% decrease in Congo red stained area (**Fig. 3.3**). These data strongly suggest that treatment with D-apoJ[113-122] peptide significantly improved cerebrovascular function in clearing Aβ through the vasculature in APP/PS1 mice.

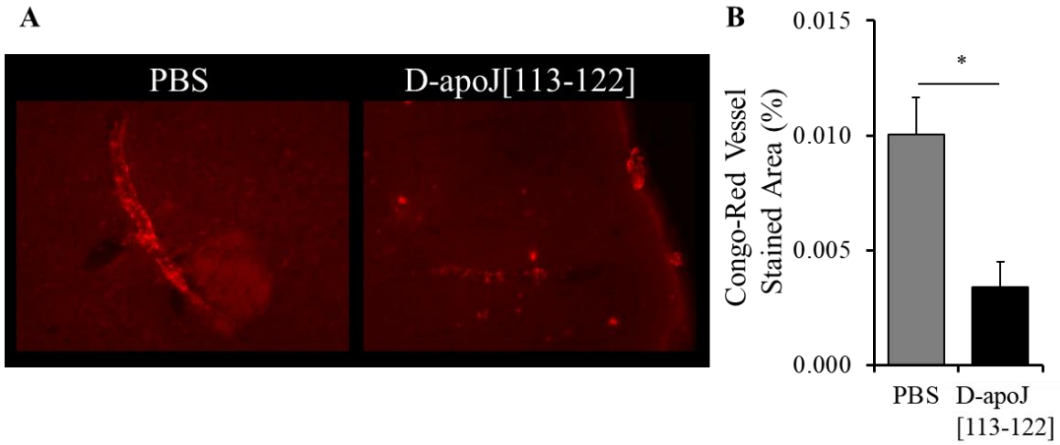

Fig. 3.3 – D-apoJ[113-122] peptide treatment reduces CAA in APP/PS1 mice. A) Representative images of Congo Red stained section. **B)** Quantification of the percent of the total vascular area stained by Congo red, representing CAA (n=9/group). * $p < 0.05$

Treatment with D-apoJ[113-122] peptide led to a significant increase in Aβ levels in the plasma:

To investigate whether the robust reduction of cerebral Aβ deposition and CAA in D-apoJ[113-122] peptide treated APP/PS1 mice resulted from an increase in clearance of brain Aβ to plasma, we measured the levels of Aβ40 and Aβ42 in the plasma by ELISA. Indeed, the results showed that the plasma level of both Aβ40 and Aβ42 were significantly increased in mice treated with D-apoJ[113-122] peptide (**Fig. 3.4**). This finding clearly indicates that D-apoJ[113-122] peptide treatment increased the clearance of Aβ from the brain to the plasma, contributing to the reduced Aβ levels in the brain of these mice. Notably, the average level of Aβ increase in the plasma corresponds well with the average level of Aβ reduction in the brain of D-apoJ[113-122] peptide treated mice, suggesting a dynamic relationship between the two pools of Aβ.

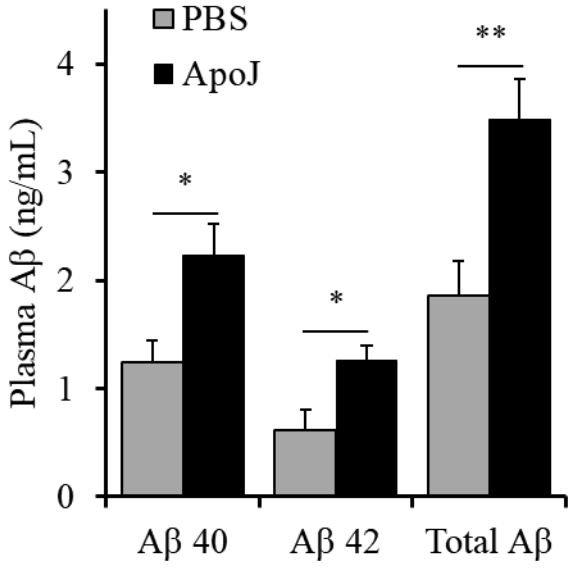

Fig. 3.4 – D-apoJ[113-122] peptide treatment leads to a significant increase in the levels of Aβ40 and Aβ42 in the plasma of APP/PS1 mice. The levels of $A\beta_{40}$ and $A\beta_{42}$ in plasma samples from APP/PS1 mice (n=9/group) were determined by ELISA. * $p < 0.05$. ** $p < 0.01$

Treatment with D-apoJ[113-122] peptide significantly improved memory retention in APP/PS1 mice:

Learning and memory deficits are a hallmark of AD and APP/PS1 mice exhibit age-related learning and memory deficits. To determine the functional outcome of D-apoJ[113-122] peptide treatment, we used the Morris water maze to assess the spatial learning and memory performance of APP/PS1 mice. The results showed that PBS-treated APP/PS1 mice display memory deficits, as expected, and that treatment with D-apoJ[113-122] peptide rescued the memory deficits, particularly in the probe trial (**Fig. 3.5**), which tests for memory retention.

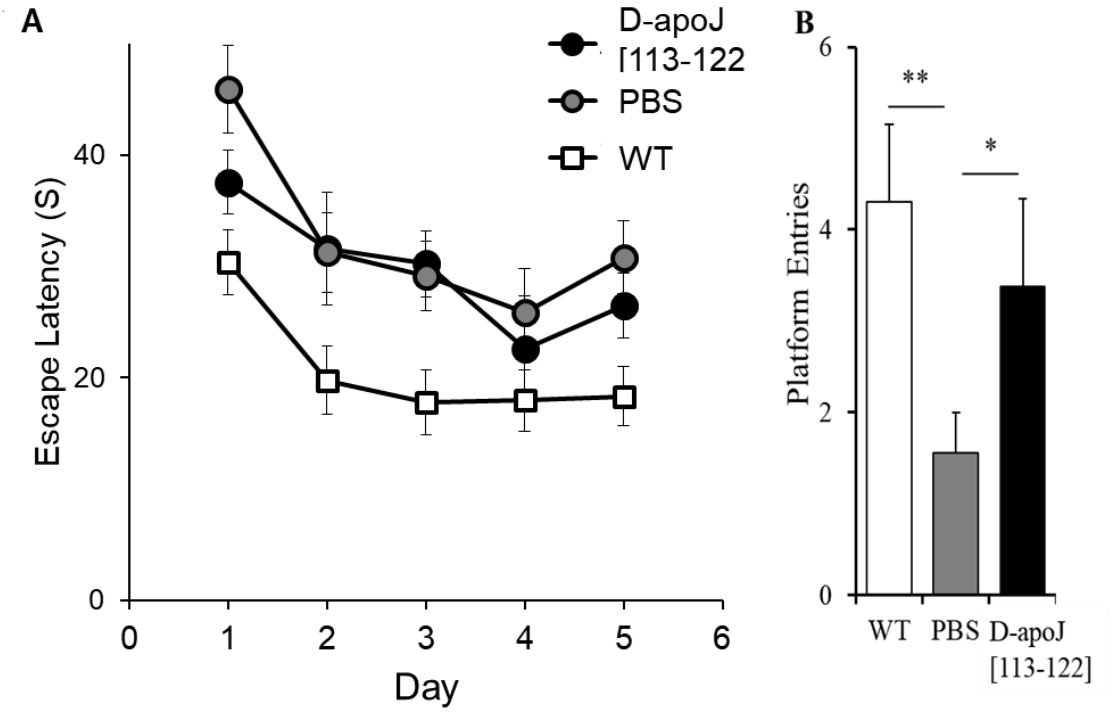

Fig. 3.5 – D-apoJ[113-122] peptide treatment rescues memory deficits in APP/PS1 mice. Performance of APP/PS1 mice (n=8-9/group age=9 mo) in the Morris water maze test after 12 weeks of treatment with apoJ[113-122] peptide or PBS. Non-Tg mice were included as WT controls (n=15 age=10 mo). **A)** Escape latency during the acquisition phase. **B)** Probe trial entries into the previous platform location. * $p < 0.05$; ** $p < 0.01$

The level of endogenous full-length apoJ was increased in D-apoJ[113-122] peptide treated APP/PS1 mice:

ApoJ has been shown to facilitate brain Aβ clearance across the blood brain barrier (Bell et al., 2006). This important role of apoJ is further supported by a recent report that a genetic deletion of apoJ shifts amyloid deposition from the brain into the vasculature, causing CAA (Wojtas et al., 2017). To examine whether D-apoJ[113-122] peptide treatment affects the level of endogenous full-length apoJ, brain tissue lysates were subjected to immunoblot analysis. The results showed that the level of apoJ was increased by 24% in D-apoJ[113-122] peptide treated APP/PS1 mice (**Fig. 3.6**). This increase of endogenous apoJ levels may have contributed to the reduction of CAA and increased clearance of Aβ from the brain to the plasma in these mice.

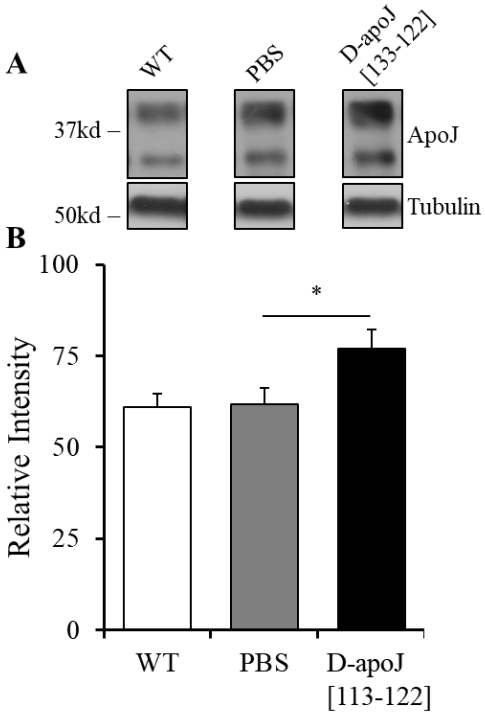

Fig. 3.6 – Increase in endogenous apoJ protein with D-apoJ[113-122] peptide treatment. A) Representative immunoblots and **B)** quantification normalized to tubulin. (n=8-9 per group). * $p < 0.05$

APP expression and processing is not affected by D-apoJ[113-122] peptide treatment in APP/PS1 mice:

To determine whether D-apoJ[113-122] peptide treatment influences the expression and processing of APP in APP/PS1 mice, brain tissue lysates were subjected to immunoblot analysis for full-length (fl) APP and the C-terminal fragments (CTFs) of APP produced from α- or β-secretase cleavage. The results showed that the level of fl-APP and CTFs of APP were not affected by the D-apoJ[113-122] peptide treatment, compared to those in PBS-treated APP/PS1 mice (**Fig. 3.7**). These results indicate that treatment with D-apoJ[113-122] peptide did not affect APP expression and processing in APP/PS1 mice, suggesting no changes in Aβ production in treated mice.

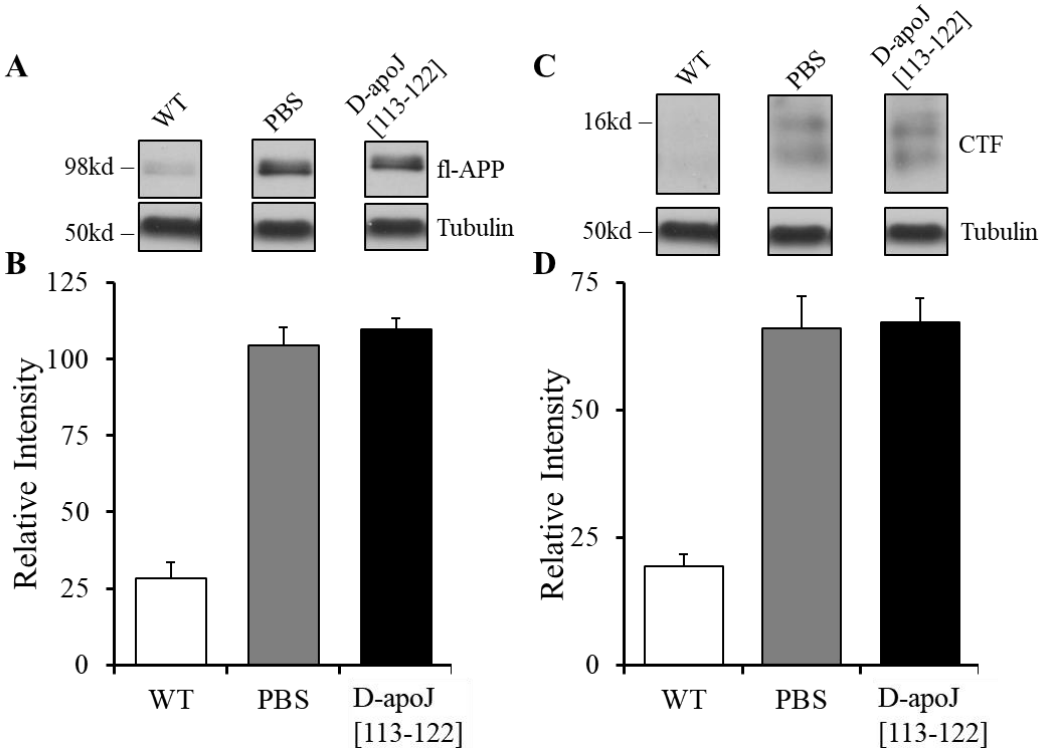

Fig. 3.7 – D-apoJ[113-122] peptide treatment does not change tg expression of fl-APP and its cleavage product, CTF, in APP/PS1 mice. A) Representative fl-APP immunoblot and **B)** quantification normalized to tubulin. **C)** Representative CTF immunoblot and **D)** quantification normalized to tubulin. (n=8-9 per group).

Peripherally administered D-apoJ[113-122] peptide had limited brain penetrance in APP/PS1 mice:

To address whether D-apoJ[113-122] peptide administered by IP injection crosses the BBB and enters the brain, plasma and brain tissue samples were collected across several time points after the last IP injection and submitted to Absorption Systems for the determination of D-apoJ[113-122] peptide concentrations. The results showed that the maximum concentrations of the peptide in plasma (~15,000 ng/ml) and brain (~50 ng/g) were reached 1 hr after injection (**Fig. 3.8**). The data indicated that less than 1% of D-apoJ[113-122] peptide in the plasma entered the brain, suggesting that D-apoJ[113-122] does not need to be present in the brain in high concentration to exert the beneficial effects described above.

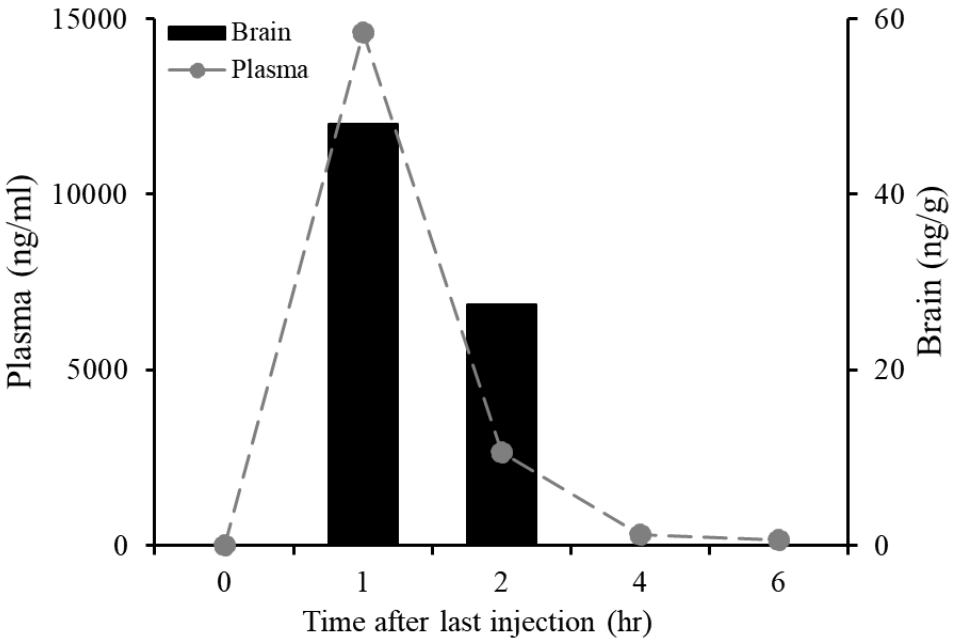

Fig. 3.8 – Kinetics of D-apoJ[113-122] peptide in plasma and brain after IP injections. The concentrations of apoJ[113-122] were determined by capillary LC-MS/MS analysis. (n=2-3 mice per time-point)

Discussion:

The great unmet need for an effective AD treatment, in addition to the existing literature on the important role of apoJ in AD, has prompted us to investigate the therapeutic potential of D-apoJ[113-122] peptide, an apoJ mimetic peptide that has cardiovascular protective effects (Navab et al., 2005). Here we report that in the APP/PS1 mouse model of AD, daily treatment with D-apoJ[113-122] peptide for 3 months results in a marked reduction of cerebral Aβ deposition and CAA, and importantly, led to a robust rescue of memory function. These convincing preclinical findings establish that D-apoJ[113-122] peptide has the potential to serve as an effective therapeutic agent for AD.

The exact mechanisms underlying the beneficial effects of D-apoJ[113-122] peptide treatment in APP/PS1 mice are not fully understood. However, our data strongly suggest that D-apoJ[113-122] peptide acts through the vasculature, promoting the clearance of Aβ from the brain to the periphery across the BBB. This notion is supported by the finding that there was a dramatic decrease in CAA in D-apoJ[113-122] peptide treated mice. CAA is the accumulation of Aβ (primarily Aβ40) (Agyare et al., 2013) in the cerebral vasculature which limits normal vascular function and can lead to cognitive decline. CAA is considered a prime cause of intracerebral hemorrhages, often with a lethal outcome in the elderly (Greenberg 1998). CAA can disturb the trafficking of Aβ out of the brain and in that way potentiate Aβ aggregation into neuritic plaques (Weller et al., 2008). Therefore, the reduction in CAA may be an important mechanism by which D-apoJ[113-

122] peptide treatment lowered brain Aβ levels and rescued cognitive function in APP/PS1 mice.

In support of the role of D-apoJ[113-122] peptide in promoting clearance of brain Aβ to the periphery across the BBB, the plasma levels of Aβ were significantly increased and negatively correlated with the brain levels of Aβ in D-apoJ[113-122] peptide treated APP/PS1 mice. It is also plausible that D-apoJ[113-122] peptide binds and stabilizes soluble Aβ in the plasma, preventing its reuptake back into the brain. Such a mechanisms is often referred to as the 'peripheral sink' and has been proposed in anti-Aβ based immunotherapies (DeMattos et al., 2001), although other immunotherapies do not seem to involve the binding of soluble plasma Aβ (Sevigny et al., 2016). From the present study, it is unclear if the increase in plasma Aβ in D-apoJ[113-122] peptide treated APP/PS1 mice is a result of enhanced efflux from the brain or limited reuptake into the brain or both. Additionally, we found a significant increase in endogenous full-length apoJ protein in brains of D-apoJ[113-122] peptide treated mice which could facilitate the clearance of Aβ.

A peripheral target for D-apoJ[113-122] peptide could best explain why the limited brain uptake of D-apoJ[113-122] peptide is not limiting the efficacy of the treatment. A peripheral site of action is more accessible to oral and IP injected D-apoJ[113-122] peptide. Another possibility is that D-apoJ[113-122] peptide modulates peripheral HDL function and enhances its anti-inflammatory properties for an extended period of time, even after D-apoJ[113-122] peptide has been cleared from the plasma. Previous studies reported that the anti-inflammatory properties of HDL from apoE-null mice given D-ApoJ[113–122]

peptide were significantly improved for at least 48 hours after a single oral dose (Navab et al., 2005). Similarly, the anti-inflammatory properties of HDL were improved for at least 24h after a single D-apoJ[113-122] peptide injection in Cynomolgus monkeys. This indicates a sustained benefit of D-apoJ[113-122] peptide even after the drug has been cleared – easing limitations normally associated with short half-life drugs. Future studies may include a more detailed analysis of the anti-inflammatory properties of HDL in D-apoJ[113-122] treated AD mice as a potential mechanism of its effect on behavioral outcomes.

The important role of HDL in AD is exemplified by several large-scale human clinical studies, which found a strong positive correlation between HDL levels and cognitive performance in late-life, as well as an inverse correlation with both AD risk and severity (recently reviewed by (Hottman et al., 2014)). Importantly, all currently understood risk factors for AD have a connection to vascular function, from an epidemiological perspective (De la Torre, 2002; Iturria-Medina et al., 2016). A recent study found that a single intravenous injection of reconstituted HDL (rHDL) reduced the levels of amyloid in the brains of APP/PS1 mice (Robert et al., 2016). In addition, a 4-week administration of rHDL was found to increase the clearance of Aβ and to improve cognitive function in AD mice (Song et al., 2014). Thus, our findings on the beneficial functions of the D-apoJ[113-122] peptide in APP/PS1 mice fit within the current literature on the role of HDL and associated lipoproteins in AD.

Our results showed that D-apoJ[113-122] peptide is capable of crossing the BBB and mediating beneficial effects in the CNS, however, penetration is limited to ~1% of the amount seen in plasma. These data suggest the drug targets of D-apoJ[113-122] peptide may in fact be peripheral or mediated through a signaling cascade that is more readily able to cross into the CNS, such as through molecules on the blood side of the BBB. Future high-throughput screenings could be a better option for detecting drug/protein interactions. It is important to note that even with very limited brain penetrance, D-apoJ[113-122] treatment led to drastic improvements in pathology and behavioral outcomes in APP/PS1 mice.

Future studies may focus on elucidating the cellular and molecular mechanisms mediating the effects of D-apoJ[113-122] peptide on Aβ efflux from the brain to the periphery. Several transporters/receptors at the BBB are known to be important in the net efflux of Aβ: PGP (Lam et al., 2001), ABCB1 (Zhang et al., 2013), BCRP/ABCG2 (Zhang et al., 2013), LRP1 (Deane et al., 2009), and RAGE (Deane et al., 2009). Other proteins are also involved in regulating Aβ transport across the BBB, including sLRP1 (Deane et al., 2009; Sagare et al., 2010), albumin (Biere et al., 1996), apoE (Martel et al., 1997), apoJ (Calero et al., 2000), transthyretin (TTR) (Sousa et al., 2007), and α2- macroglobulin (α2M) (Narita et al., 1997). Identification of the exact molecular pathways through which D-apoJ[113-122] peptide operates will allow more precise drug targeting in the future.

In conclusion, the present study provides preclinical evidence that treatment with the D-apoJ[113-122] peptide through peripheral administration markedly reduced amyloid

pathology and rescued cognitive function in APP/PS1 mice, most likely through the attenuation of CAA and the increase of Aβ clearance from brain to plasma. These findings strongly suggest that D-apoJ[113-122] peptide could be a potential therapeutic agent to mitigate cognitive impairment and amyloid pathology in AD.

CHAPTER 4 – SYSTEMIC OR FOREBRAIN NEURON SPECIFIC DEFICIENCY OF GERANYLGERANYLTRANSFERASE-1 IMPAIRS SYNAPTIC PLASTICITY AND REDUCES DENDRITIC SPINE DENSITY

Introduction:

Proteins can undergo several different types of posttranslational modifications which result in proper tertiary structure, function, and subcellular location (Krishna and Wold 1993). One important posttranslational modification is prenylation, (Lane and Beese 2006) which is the process of adding short-chain lipid molecules (isoprenoids) to target proteins via an irreversible covalent bond. Isoprenoids are intermediates in the mevalonate/cholesterol biosynthesis pathway (Goldstein and Brown 1990) (**Fig. 4.1**).

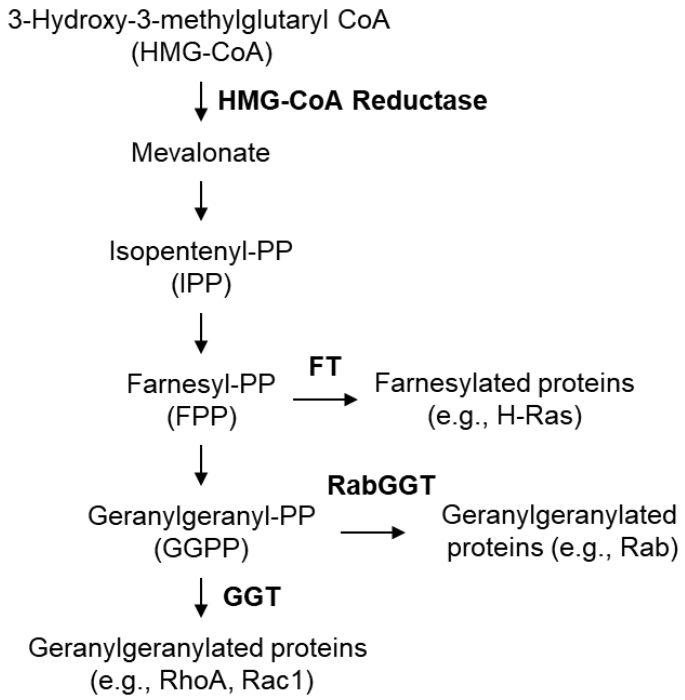

Fig. 4.1 – Isoprenoid synthesis and protein prenylation pathways.

The two major isoprenoids are the 15-carbon FPP and the 20-carbon GGPP. FPP and GGPP are substrates of FT and GGT, which respectively, catalyze the attachment of farnesyl or geranylgeranyl group to target proteins with the CAAX motif (McTaggart 2006). A third prenyltransferase, geranylgeranyl tranfersase-2 (RabGGT), also uses GGPP as its substrate to prenylate target proteins. Over 100 proteins are known to undergo prenylation, included heterotrimeric G-protein subunits and nuclear lamins (McTaggart 2006; Berndt et al., 2011), but notably the largest and most well studied group of prenylated proteins is the Ras superfamily of small GTPases such as Ras, Rho, and Rab proteins. These small GTPases serve as molecular switches and regulate a plethora of cellular processes and functions, including dendritic spine morphogenesis and synaptic plasticity (Hottman and Li 2014). The importance of protein prenylation is further underscored by the findings that germline deletion of FT or GGT is embryonically lethal (Sjogren et al., 2007; Mijimolle et al., 2005), and dysregulation of prenylated proteins causes cancers and a number of other diseases including cardiovascular and cerebrovascular diseases, bone diseases, progeria, and potentially neurodegenerative diseases such as AD (McTaggart 2006; Li et al., 2012; Gao et al., 2016).

We previously reported that haplodeficiency of either GGT or FT reduced Aβ accumulation in a transgenic mouse model of AD (Cheng et al., 2013b). However, only FT haplodeficiency rescued cognitive function in these animals. GGT haplodeficiency similarly reduced amyloid plaques and reduced neuroinflammation, but was not sufficient to rescue memory function of the animals. As geranylgeranylaed Rho family proteins are crucial in synapse/spine formation and remodeling (Tolias and Duman 2011), we

hypothesized that GGT deficiency might have detrimental effects that could neutralize the benefits of attenuated AD-related neuropathology. The current study was undertaken to address the impact of GGT deficiency on dendritic spine density and synaptic plasticity, the cellular basis of learning and memory formation (McGaugh 2000). Our results showed that either germline GGT haplodeficiency or forebrain neuron-specific GGT deficiency reduced the magnitude of hippocampal LTP and decreased the dendritic spine density of cortical neurons in mice. These findings corroborate the pivotal role of GGT in the development and maintenance of neurophysiological function of the brain.

Materials and methods:

Animals:

Germline/systemic GGT-haplodeficient (GGT+/-) mice have been described previously (Liu et al., 2010; Cheng et al., 2013a). The forebrain neuron-specific GGT deficient mice were generated by breeding the GGT floxed *(*$GGT^{f/f}$) mice (Sjogren et al., 2007) with a αCaMKII promote-driven Cre recombinase (Cre+) mice (Tsien et al., 1996). Further interbreeding of resulting siblings with genotypes of $GGT^{f/+}$Cre+ and $GGT^{f/+}$Cre- produced $GGT^{f/f}$Cre+ and $GGT^{f/f}$Cre- (wild-type, WT) mice, which were used in this study. All genotypes were determined using DNA extracted from tail biopsies and amplified via PCR using gene-specific primers. The average mouse age was 8-12 mo and both male and female were used. Littermate controls were used whenever possible. All animal procedures in this study were prospectively reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota.

Electrophysiology:

Mice were anesthetized using isoflurane which was confirmed with a foot pinch to be followed by decapitation as previously described (Parent et al., 2014). Briefly, brains were collected and cooled in "cutting solution" containing (mM): 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 7 Dextrose, and 240 sucrose (Sigma). Transverse hippocampal slices (400μm) were prepared using a vibratome (Leica) while immersed in ice cold cutting solution. Cut sections were allowed to recover for 1 to 4 h in artificial cerebrospinal fluid (aCSF) containing (mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$, and 25 dextrose (pH 7.4) with constant bubbling of 95% O_2/5% CO_2.

After recovery, slices were placed into the recording chamber (Automate Scientific, Berkeley, CA) with aCSF flowing at approximately 1.5 ml/min at 28-30C. A presynaptic stimulation was delivered to the CA3/CA1 boundary of the hippocampus using a bipolar tungsten-stimulating electrode (FHC) driven by A365R constant-current stimulus isolators (World Precision Instruments, Sarasota, FL). Stimulation intensity was adjusted up to a maximum of 95μA. Recording electrodes were carefully placed in the CA1 of the hippocampus. Evoked field excitatory post-synaptic potentials (fEPSPs) were recorded with pulled borosilicate glass micropipettes filled with aCSF using a two channel 700B multiclamp amplifier, 1440A Digidata DA/AD converter, and pClamp data acquisition software (Molecular Devices, Sunnyvale, CA). The stimulation intensity was adjusted to elicit a fEPSP response of 0.6–0.8 mV for 20 minutes to produce a stable baseline. Once a

sufficiently stable baseline was established, LTP was induced using two theta burst stimulations with a 20 second interval. The fEPSP slope recordings from the 35-40 minute post-induction time points were normalized to the baseline fEPSP slope values and compared between groups. External noise was reduced via a faraday cage, aluminum shielding, with a 50/60 Hz noise canceling Humbug hardware (Quest Scientific, North Vancouver, BC, Canada). Basal paired-pulse facilitation (PPF) was analyzed during the baseline recordings of LTP experiments. Two stimulations were given in a quick 30ms succession and the amplitudes for each fEPSP were recorded and a ratio of these amplitudes yielded the basal PPF ratio which was compared between groups.

Input/Output (I/O) curves were generated from similarly prepared slices. Slices were subjected to 5 stimulations per intensity ranging from 0µA up to 150µA in 10µA intervals. The fEPSP magnitudes were averaged for each stimulation intensity then compared across groups using a repeated measures ANOVA.

Tissue collection and preparation:

Tissue collection protocols were followed as previously mentioned (Cheng et al., 2013a). Briefly, mice were deeply anesthetized and blood was collected by cardiac puncture with heparinized needles. Following perfusion with ice-cold PBS, brain hemispheres were removed and fixed in 4% paraformaldehyde or snap frozen in liquid nitrogen. Brain homogenates were prepared as we described previously (Cao et al., 2007; Lewis et al., 2010).

Immunoblot analysis:

For immunoblot analysis, equal aliquots of membrane and cytosolic fractions were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with primary antibodies (all from Santa Cruz Biotechnology, Dallas, TX) against the following: RhoA (26C4, sc-418), Rac1 (C-14, sc-217), H-RAS (C-20, sc-520), and Calnexin (H-70, sc-11397). Overnight primary antibody incubation was followed by incubation with HRP-conjugated secondary antibodies. Signal was detected by the ECL Plus Western Blotting System (GE Healthcare) and quantified using ImageJ software. For confirmation of membrane and cytosolic fractionation, the blots were stripped and re-probed with antibodies against a membrane protein, calnexin (sc-11397), and a cytosolic protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (V-18, sc-20357), respectively.

Golgi staining and analysis of dendritic spine density:

Based on a previous method (Dumanis et al., 2009), mice brains were freshly dissected and processed for Golgi staining using the FD Rapid GolgiStain^{TM} kit (Cat. #: PK401A, FD NeuroTechnologies, Inc.). Golgi staining followed all manufactures' instructions. Bright-field microscopy images (100x) were taken of pyramidal neurons in cortical layers II/III. Spine linear density was measured using Image Pro Plus software. Fifteen neurons were randomly selected from each animal and five apical oblique (AO) and five basal shaft (BS) dendrites were chosen from tertiary dendritic segments of each neuron. Spine densities were quantified and compared across groups.

Preparation of membrane and cytosolic fractions:

Cortical samples were homogenized in an ice cold, low osmotic lysis buffer containing 5 mMTris-HCL (pH 7.4), 2mM EDTA, 1x protease inhibitors (Roche Applied Science, Indianapolis, IN), and 1x phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Homogenates were centrifuged at 1500 x *g* for 15 min at 4°C to remove tissue debris. The clear supernatant was transferred to a new tube and further centrifuged at 100,000 x *g* for 60 min at 4°C in a Beckman Coulter Optima^{TM} MAX-XP Tabletop Ultracentrifuge. The resulting supernatant was collected as the cytosolic fraction without disturbing the pellet. The pellet was washed twice with lysis buffer by centrifugation at 100,000 x g for 15 min at 4°C. Finally, the pellet was resuspended in lysis buffer of original volume and this fraction was used as the membrane fraction.

Statistical analysis:

All data are expressed as means ± standard errors of the means (SE). Comparison of different genotype groups was performed by Student's t test and repeated measures analysis of variance (ANOVA). $p < 0.05$ was considered statistically significant. Both male and female mice were included in the study. Littermates were used whenever possible. Investigators were blinded to genotypes of the mice during the experiments.

Results:

Hippocampal synaptic plasticity is impaired in GGT haplodeficient mice:

Our previous work demonstrated that compared to WT, GGT haplodeficient (GGT+/-) mice express ~50% GGT protein content (Cheng et al., 2013b). GGT activity and prenylation of Rho GTPases has a major effect on synaptic turnover and stability (Newey et al., 2005; Kiraly et al., 2010). To assess the impact of GGT haplodeficiency on neuroplasticity, we performed a series of electrophysiological experiments at the hippocampal Schaffer collateral-CA1 synapses in acute brain slices.

First, the basal synaptic transmission was measured by compilation of I/O curves (Carvalho and Buonomano 2009) (**Fig. 4.2a**), which shows the fEPSP amplitude responding to increasing stimulus intensities. There was a trend decrease (but did not reach statistical significance by repeated measures ANOVA) in the average fEPSP amplitudes for a given stimulation intensity in GGT+/- mice compared to their WT littermates. This result indicates that there is no significant reduction in basal signal propagation in the brain of GGT+/- mice.

Next, the short-term presynaptic plasticity was measured by examining the PPF ratios, which tests the ability of two successive stimuli to elicit an increased post-synaptic response. The mechanisms underlying PPF are exclusively pre-synaptic (Isaac et al., 1998). PPF is a function of presynaptic release probability due to increased presynaptic Ca^{2+} concentration leading to a greater release of the excitatory neurotransmitter, glutamate, to the post-synaptic cell (Dudel and Kuffler 1961; Del Castillo and Katz 1954; Zucker and

Regehr 2002). The results showed that the basal PPF at a 30ms inter-stimulus interval was significantly reduced in GGT+/- mice compared to WT littermates (**Fig. 4.2b**). This finding suggests that GGT haplodeficiency causes a decrease in synaptic vesicle release probability from the pre-synaptic terminals.

Finally, the magnitude of LTP was measured. LTP in the hippocampus is thought to be the cellular mechanism underlying learning and memory and is defined as the long-lasting increase in signal transmission between two neurons (Fitzjohn et al., 2001; Collingride and Bliss 1993). The results showed that the magnitude of was severely reduced in GGT+/- mice compared to their wild-type littermates (**Fig. 4.2c and 4.2d**). Taken together, these electrophysiological data demonstrate that germ-line GGT haplodeficiency impairs both presynaptic and postsynaptic plasticity.

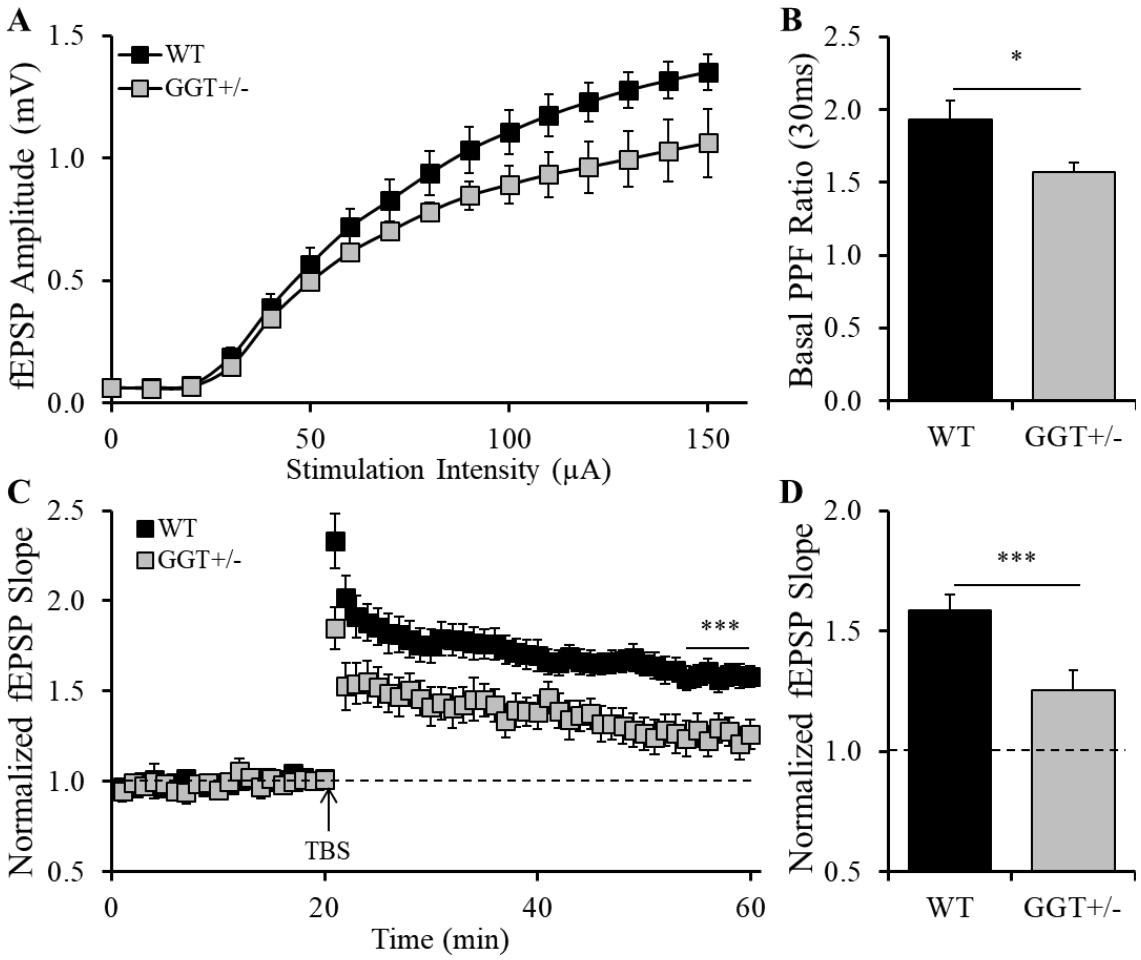

Fig. 4.2 – Short- and long-term synaptic plasticity is impaired in GGT+/- mice. A) Input/Output (I/O) curves. The field excitatory post-synaptic potentials (fESPS) were recorded and averaged from 5 stimulations per intensity ranging from 0µA up to 150µA in 10µA intervals in hippocampal slices from GGT+/- and WT mice (n = 15-25 slices/5 mice per genotype). **B)** Paired-pulse facilitation (PPF) ratios. Basal PPF responses were quantified from baseline recordings during LTP experiments in hippocampal slices frm GGT+/- and WT mice (n = 21 slices/5-7 mice per genotype). Two stimulations were performed in a quick 30ms succession. The amplitudes for each fESPS were recorded and the PPF ratio was expressed as the ratio of the responses from the stimulations. **C) and D)** Long-term potentiation (LTP). Following a 20-min stable baseline, the LTP was induced by a theta burst stimulation (TBS) protocol with a 20 second interval and the fESPS recorded. The magnitude of LTP was expressed as the slopes of the rising phase of the fEPSPs normalized to baseline in hippocampal slices from GGT+/- and WT mice (n = 24-32 slices/9-10 mice per genotype. The LTP magnitude of the last 5 minutes (35-40 minute post-induction) was averaged for each genotype of mice. *$P < 0.05$; *** $P < 0.001$.

Dendritic spine density of cortical neurons is markedly reduced in GGT haplodeficient mice:

Following the electrophysiological experiments, we attempted to explore the cellular and structural basis for the reduced synaptic plasticity observed in GGT+/- mice. Small GTPases that are geranylgeranylated by GGT play critical roles in regulating dendritic spine formation/pruning (Tolias and Duman 2011; Yuan et al., 2015). Therefore, we used a Golgi staining method and quantified dendritic spine density in cortical neurons. The results showed that there was a substantial decrease in both apical and basal dendritic spine counts in GGT+/- mice compared to WT littermate controls, indicating impaired spine genesis and/or reduced basal synaptic stability in GGT+/- mice (**Fig. 4.3**). Since spine formation correlates with long-term synaptic plasticity and memory (M Segal 2005), our results strongly suggest that the reductions in synaptic plasticity in GGT+/- mice are a result of decreases in dendritic spine genesis or stability.

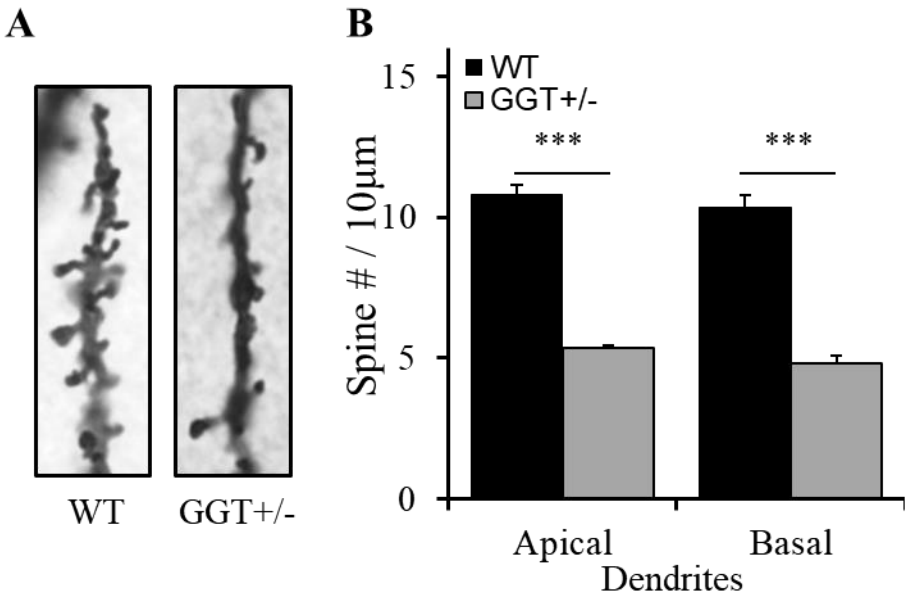

Fig. 4.3 – Dendritic spine density is decreased in pyramidal neurons in cortical layers II/III of GGT+/- mice. A) Representative images of dendrites in Golgi-stained brain sections from GGT+/- and WT mice (n=4 mice/genotype). **B)** Average spine densities on apical oblique and basal shaft dendrites of pyramidal neurons (n = 75 apical or basal dendritic segments/15 neurons per mouse). *** $P < 0.001$.

Membrane association of geranylgeranylation-targeted small GTPases is reduced in forebrain neuron-specific GGT deficient mice:

The well-known targets of GGT are the Rho family of small GTPases, including Rac1 and RhoA which play pivotal roles in dendritic morphogenesis and synaptic plasticity (Kimberly and Duman 2011; Newey et al., 2005). These proteins are extensively geranylgeranylated by GGT under normal physiological conditions and this lipid modification is required for their proper membrane association and function. To determine the impact of neuron-specific GGT deletion on cellular location of small GTPases in the brain of $GGT^{f/f}$Cre+ mice, the tissue lysate of cerebral cortex was subjected to subcellular fractionation by ultracentrifugation, followed by immunoblot analysis of Rac1, RhoA, and H-Ras. The results showed that the levels of membrane-associated (geranylgeranylated)

Rac1 and RhoA were significantly reduced, while their levels in cytosolic (un-geranylgeranylated) fractions increased in GGT$^{f/f}$Cre+ mice compared to GGT$^{f/f}$Cre- littlemates (**Fig. 4.4**). Importantly, the cellular distribution of H-Ras, an exclusively farnesylated small GTPase (Zhang and Casey 1996), was unaffected (**Fig. 4.4**), indicating that the specific deletion of GGT only reduced geranylgeranylation of target proteins not farnesylation of other proteins. In addition, these findings validated that the reduction of protein geranylgeranylation could be readily detected in the tissue lysate of the cerebral cortex even though only neuronal GGT was deleted in GGT$^{f/f}$Cre+ mice. As geranylgeranylation plays a critical role in the cellular trafficking and function of Rac1 and RhoA, reduced geranylgeranylation of these small GTPases most likely underlies the impairment of dendritic spine genesis and synaptic plasticity in GGT-deficient mice.

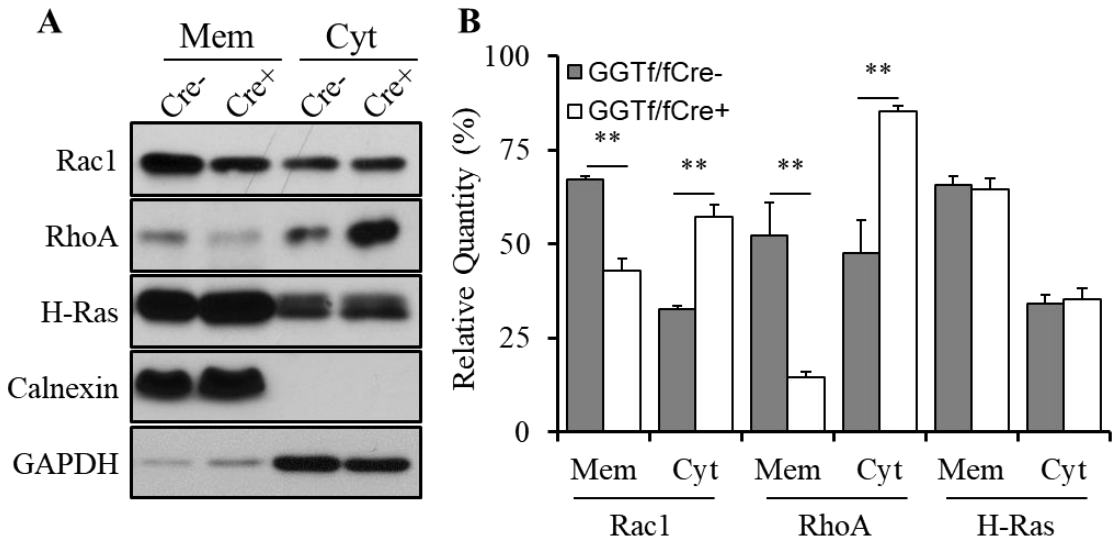

Fig. 4.4 – Subcellular distribution of selected small GTPases is disrupted in the brain of GGT$^{f/f}$Cre+ mice. Forebrain tissue homogenates from GGT$^{f/f}$Cre+ and GGT$^{f/f}$Cre- mice were subjected to ultracentrifugation for the preparation of membrane (Mem) and cytosolic (Cyt) fractions (n = 3 mice/genotype). **A)** Representative images from immunoblot analyses for Rac1, RhoA, and H-Ras. Calnexin and GAPDH were used as markers for membrane-associated and cytosolic proteins, respectively. **B)** Densitometric quantification of immunoblot analysis, showing relative distribution of each protein in the membrane (prenylated) and cytosolic (unprenylated) fraction. **P < 0.01.

The magnitude of hippocampal long-term potentiation is modestly reduced in forebrain neuron-specific GGT deficient mice:

To avoid any potential influence of germ-line GGT haplodeficiency during embryonic development and to determine the specific role of neuronal GGT in adult brain, we generated forebrain neuron-specific GGT knockout ($GGT^{f/f}$Cre+) mice and their WT littermates ($GGT^{f/f}$Cre-) by crossbreeding $GGT^{f/f}$ mice with CaMKIIα-Cre mice (Tsien et al., 1996). Since CaMKIIα-Cre is only expressed in neurons of the adult forebrain, the essential function of GGT during embryonic development is preserved in these mice. $GGT^{f/f}$Cre+ mice were subjected to the same sets of electrophysiological experiments as in GGT+/- mice.

The results from the I/O curves showed there was no difference in the average fEPSP amplitudes for a given stimulation intensity in the brain slices of $GGT^{f/f}$Cre+ mice compared to their $GGT^{f/f}$Cre- littermates (**Fig. 4.5a**). Similar I/O responses observed in $GGT^{f/f}$Cre+ and $GGT^{f/f}$Cre- indicate that forebrain neuron-specific GGT deficiency does not affect basal transmission.

Similarly, comparison of PPF ratios showed no significant differences between $GGT^{f/f}$Cre+ mice and $GGT^{f/f}$Cre- littermates (**Fig. 4.5b**). These results suggest that $GGT^{f/f}$Cre+ mice do not exhibit a decrease in the probability of synaptic vesicle release from the pre-synaptic terminal. Therefore, it appears that forebrain neuron-specific GGT deficiency does not significantly affect presynaptic plasticity, unlike the situation in GGT+/- mice, in which presynaptic plasticity was compromised.

In the experiments for long-term synaptic plasticity, we found that the magnitude of LTP was reduced in GGT$^{f/f}$Cre+ mice compared to GGT$^{f/f}$Cre- littermates (**Fig. 4.5c and 4.5d**). Interestingly, the reduction in the magnitude of LTP in GGT$^{f/f}$Cre+ mice was less severe than that in GGT+/- mice, relative to their respective WT littermate controls. These results suggest that the normal level of GGT in both neurons and non-neuronal cells is important in maintaining long-term synaptic plasticity.

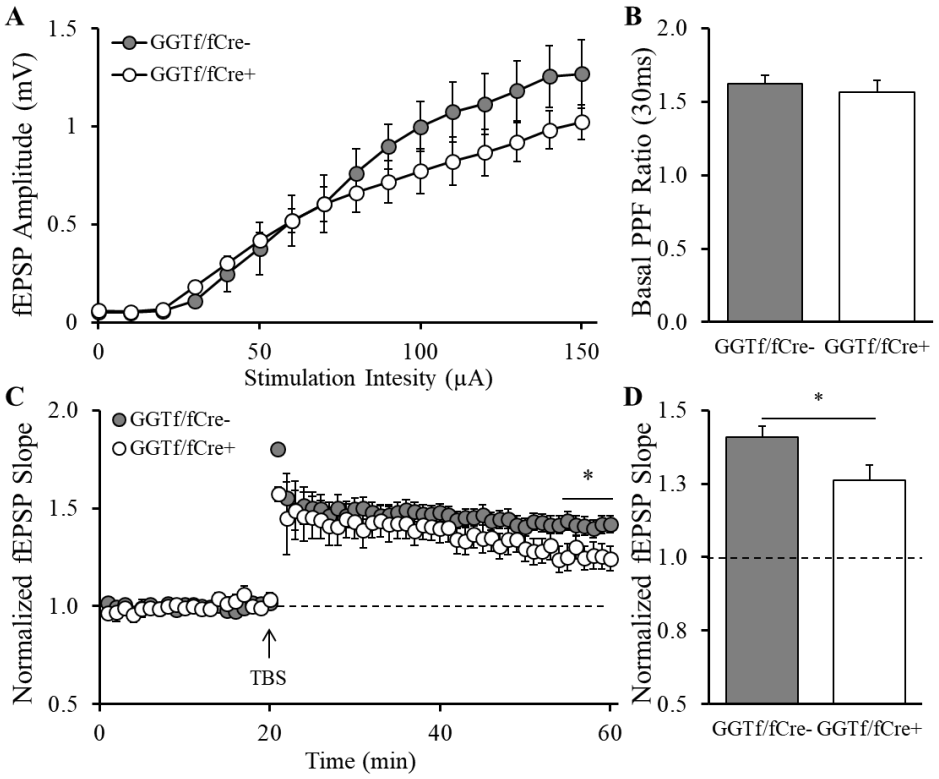

Fig. 4.5 – Long-term synaptic plasticity is impaired in GGT$^{f/f}$Cre+ mice. A) Input/output (I/O) curves. The field excitatory post-synaptic potentials (fEPSPs) were recorded and averaged from 5 stimulations per intensity ranging from 0μA up to 150μA in 10μA intervals in hippocampal slices from GGT$^{f/f}$Cre+ and GGT$^{f/f}$Cre- mice (n = 17-19 slices/4 mice per genotype). **B)** Paired-pulse facilitation (PPF) ratios. Basal PPF responses were quantified from baseline recordings during LTP experiments in hippocampal slices from GGT$^{f/f}$Cre+ and GGT$^{f/f}$Cre- mice (n = 17-20 slices/9 mice per genotype). Two stimulations were performed in a quick 30ms succession. The amplitudes for each fEPSPs were recorded and the PPF ratio was expressed as the ratio of the responses from the stimulations. **C)** and **D)** Long-term potentiation (LTP). Following a 20-min stable baseline, the LTP was induced by a theta burst stimulation (TBS) protocol with a 20-sec interval and the fEPSPs recorded. The magnitude of LTP was expressed as the slopes of the rising phase of the fEPSPs normalized to baseline in hippocampal slices from GGT$^{f/f}$Cre+ and GGT$^{f/f}$Cre- mice (n = 18-24 slices/10 mice per genotype. The LTP magnitude of the last 5 minutes (35-40 minute post-induction) was averaged for each genotype of mice. *P < 0.05.

Dendritic spine densities in cortical neurons are reduced in forebrain neuron-specific GTT deficient mice:

To address whether the changes in dendritic spine densities underlie the reduced LTP magnitude in $GGT^{f/f}$Cre+ mice, we quantified tertiary apical and basal dendrites from cortical neurons. We found that both apical and basal dendritic spine counts were decreased in cortical neurons from $GGT^{f/f}$Cre+ mice compared to $GGT^{f/f}$Cre- littermates, indicating the importance of neuronal GGT for maintaining normal dendritic spine densities (**Fig. 4.6**). Consistent with the reduction in the magnitude of LTP, the reduction of dendritic spine densities in $GGT^{f/f}$Cre+ mice was to a lesser degree than that in GGT+/- mice, relative to their respective WT littermate controls. These results further suggest that the normal level of GGT in both neurons and non-neuronal cells is crucial to maintain dendritic spine density and synaptic plasticity.

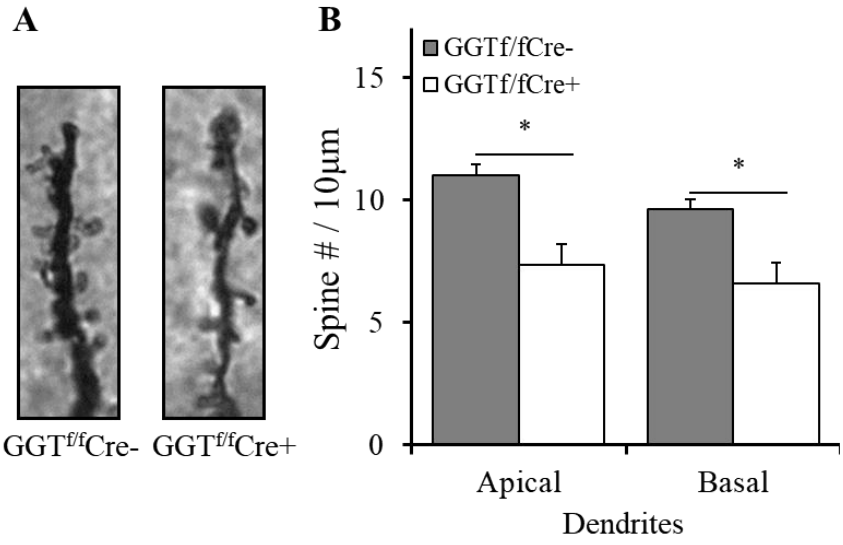

Fig. 4.6 – Dendritic spine density is decreased in pyramidal neurons in cortical layers II/III of $GGT^{f/f}$Cre+ mice. A) Representative images of dendrites in Golgi-stained brain sections from $GGT^{f/f}$Cre+ and $GGT^{f/f}$Cre- mice (n=3-4 mice/genotype). **B)** Average spine densities on apical oblique and basal shaft dendrites of pyramidal neurons (n = 75 apical or basal dendritic segments/15 neurons per mouse). * $P < 0.05$.

Discussion:

The role of GGT in the nervous system has been explored previously. Gao et al has concisely summarized recent findings on the role of GGT-mediated prenylation in the brain (Gao et al., 2016). However, the majority of the previous studies used the pharmacological approaches to manipulate the activity of GGT. While statins and GGT inhibitors are valuable pharmacological tools to probe prenylation pathways, there are limitations. Statins inhibit the activity of HMG-CoA reductase and thus decease the production of isoprenoids instead of directly inhibiting the activity of a specific prenyltransferase. Statin-induced limitation of isoprenoids (FPP and GGPP) could affect both farnesylation and geranylgeranylation by all prenyltransferases rather than GGT alone. In addition, prenylation-independent effects of isoprenoids and cholesterol further complicate the impact of treatment with statins. Although synthetic inhibitors targeting GGT have facilitated some of the experiments, potential side effects and the poor blood-brain barrier permeability limit the application of these inhibitors for long-term studies *in vivo*. Here we took the genetic approach and generated conditional forebrain neuron-specific GGT deficient mice, as well as using germline GGT haplodeficient mice, to better understand the role of GGT in synaptic function.

We found that there were no significant changes in basal synaptic transmission in either GGT+/- or $GGT^{f/f}$Cre+ mice, evidenced by similar I/O curves from different genotypes. These results indicate that these mice similar post-synaptic responses compared to WT mice (Campanac and Debanne 2008; Marder 2004; Staff and Spruston 2003;

Daoudal and Debanne 2003; Carvalho and Buonomano 2009). In the experiments measuring the presynaptic short-term plasticity, we found that hippocampal PPF ratios were reduced in GGT+/- mice, but not in $GGT^{f/f}$Cre+ mice. Changes in PPF ratio indicate a reduced ability for presynaptic neurons to release neurotransmitter to the post-synaptic cell. PPF changes are exclusively pre-synaptic and can be caused by several mechanisms (Del Castillo and Katz 1954; Dudel and Kuffler 1961; Zucker and Regehr 2002), such as increased Ca^{2+} sequestration, misaligned cytoskeletal features that reduce the efficiency of vesicle release, or increased inhibitor signaling from GABAergic neurons. It is not clear which mechanisms were responsible for the reduced PPF ratios in GGT+/- mice. Interestingly, such deficits in PPF did not occur in $GGT^{f/f}$Cre+ mice, suggesting that the changes in short-term plasticity might be a result of altered neuronal development caused by a systemic/germline reduction of GGT in GGT+/- mice. This notion is supported by the importance of GGT during early development as a complete KO is embryonic lethal (Liu et al., 2010; Sjogren et al., 2007).

The induction and maintenance of LTP in the hippocampus is widely considered to be the cellular/synaptic correlate of learning and memory (McGaugh 2000). We found that the magnitude of LTP was reduced in both GGT+/- and $GGT^{f/f}$Cre+ mice, indicating the importance of both systemic and neuronal GGT in long-term synaptic plasticity. Intriguingly, the deficit in LTP was more severe in GGT+/- mice than that in $GGT^{f/f}$Cre+ mice compared to their respective WT littermates. This observation further suggests the critical role of normal GGT levels during early development and for synaptic function in mature brain. Germline haplodeficiency of GGT led to a significant reduction in both short-

term and long-term synaptic plasticity. Our results corroborate some of the previous findings using GGT inhibitors. It has been shown that while acute treatment with a GGT inhibitor in hippocampal slices from WT mice does not affect the induction and maintenance of LTP (Kotti et al., 2008; Mans et al., 2012), long-term treatment with a GGT inhibitor reduces the magnitude of LTP (Kotti et al., 2008).

Compelling evidence shows that dendritic spines are morphological building blocks of long-term plasticity and memory (Segal 2005, 2017). Our study showed that dendritic spine densities in cortical neurons were significantly reduced in both GGT+/- and $GGT^{f/f}$Cre+ mice. Consistent with our results from LTP experiments, the reduction of dendritic spine density was more marked in GGT+/- mice than in $GGT^{f/f}$Cre+ mice compared to their respective WT littermate controls. Our results from these mice are also in line with previous findings from studies using pharmacological and transgenic approaches. Using an inhibitor of GGT *in vitro* and transgenic expression of a dominant-negative mutant GGT *in vivo*, Luo et al reported that GGT might be a signaling molecule itself and GGT-mediated protein prenylation could play an important role in regulating neuromuscular synapse formation and/or maintenance (Luo et al., 2003). Subsequent studies showed that activation of GGT is required for activity- and BDNF–dependent dendritic morphogenesis and synaptogenesis in cultured hippocampal neurons and cerebellar Purkinje cells (Zhou et al., 2008; Wu et al., Luo 2010; Li et al., 2013). Further, inhibition of GGT activity by lateral ventricular injection of a GGT inhibitor was found to decrease dendritic spine density in the hippocampus of treated mice (Yuan et al., 2015). Others have also identified GGT as a significant player in the regulation of neurite/dendritic outgrowth and synaptic markers

(Samuel et al., 2014; Li et al., 2016; Moutinho et al., 2016). However, not all results are consistent. For example, Samuel et al have shown that inhibition of geranylgeranylation increases neurite branching in cultured neurons (Samuel et al., 2014). Li et al recently reported that protein prenylation acts as an endogenous brake on axonal growth (Li et al., 2016). Through high-throughput drug screening, they identified statins as the most effective molecules to enhance neurite outgrowth of different types of neurons in culture and demonstrated that inhibition of protein prenylation accounted for the statin-induced increase in axonal growth. They further showed that the expression level of GGT, but not FT, was elevated in motor neurons of patients with early-onset ALS, suggesting that protein prenylation inhibitors might have therapeutic potential to accelerate neuronal regeneration. It is worth noting that this elevation of GGT expression only occurs under the pathological condition of early-onset ALS and specifically in motor neurons (Li et al., 2016). Thus, inhibition of GGT in motor neurons of early-onset cases of ALS to normal level of GGT could be beneficial. However, here we show that under physiological conditions, germline/systemic or forebrain neuron-specific reduction of GGT results in detrimental effects on synaptic plasticity and dendritic spine density. These findings indicate that a proper level of GGT expression/activity is required for normal structure and function of neurons during development and in mature brain.

It is well known that Rho GTPases are major regulators of synaptic plasticity, dendritic growth, and spine morphogenesis (Kimberly and Duman 2011; Newey et al., 2005; Govek et al., 2011). These small GTPases primarily undergoes geranylgeranylation for their proper cellular localization and interactions with their downstream effector

proteins. In particular, the role of major Rho proteins, such as Rac1 and RhoA, in neuronal structure and function has been extensively studied (Kimberly and Duman 2011; Newey et al., 2005). Activation of Rac1 promotes dendritic arborization, remodeling, and synapse formation, whereas activation of RhoA exhibits opposite functions, reducing dendritic complexity and spine density (Newey et al., 2005). Thus, Rac1 and RhoA have a crucial checks-and-balances role to ensure the plasticity of dendritic structure and function. Our study provides evidence that GGT deficiency in neurons caused a significant reduction in the membrane association (geranylgeranylation) of Rac1 and RhoA in the brain of $GGT^{f/f}$Cre+ mice. Such inadequate geranylgeranylation-induced improper localization of Rac1 and RhoA which is expected to impair their interactions with effector proteins and disrupt the balance of their functions, causing dendritic spine destabilization. Indeed, the critical role of prenylated Rac1 in dendritic morphogenesis has been shown previously, in which deletion or mutation of the geranylgeranylation site in Rac1 fails to mediate the beneficial effects of GGT overexpression on dendritic arborization in cultured neurons (Zhou et al., 2008). Using a similar approach, another study reported that expression of a non-prenylated Rac1 led to abnormal cell morphology and neurite initiation because of aberrant activation of cytosolic signaling pathways (Reddy et al., 2015). Therefore, reduction of dendritic spine density and synaptic plasticity in GGT-deficient mice most likely result from inadequate geranylgeranylation and dysfunction of these Rho proteins. Interestingly, a recent study found that GGT activity and protein/gene expression levels were significantly decreased in the brain of aged mice compared to the brain of young mice (Afshordel et al., 2014). Consistently, membrane-associated (geranylgeranylated) RhoA

and Rac1 levels were reduced in the aged mouse brains. These findings suggest that GGT/protein geranylgeranylation may play an important role in brain aging and that GGT-deficient mice may serve as a model of accelerated brain aging. Clearly, GGT target proteins are not limited to Rac1 and RhoA. To fully assess the scope of geranylgeranylated proteins affected by GGT deficiency, an unbiased prenylomic analysis will be required. Currently such a prenylomic approach has been applied for studies *in vitro* and is actively pursued for studies *in vivo* (Palsuledesai et al., 2016).

The present study demonstrates that germline/systemic or forebrain neuron specific deficiency of GGT reduces dendritic spine density and impairs synaptic plasticity in the brain of young adult mice, concurrently with reduced geranylgeranylation of Rho proteins. These results closely resemble changes found in the brain of aged mice, suggesting an important role of GGT and its target proteins in normal brain aging. While the use of GGT inhibitors could be beneficial under pathological conditions with over activation or upregulation of GGT, our findings caution potential detrimental effects on synaptic function from the chronic use of GGT inhibitors.

CONCLUDING REMARKS AND PERSPECTIVES:

In the past 20 years, much progress has been made on understanding the symptoms, etiology and pathogenic mechanisms of AD. However, to date there is no effective prevention or treatment for this debilitating disease. It is clear that cognitive impairment is the earliest symptom in AD. Compelling evidence suggests that HDL could be a viable target for developing therapeutic strategies to mitigate cognitive deficits in AD. However, several important issues need to be addressed. First, the level of plasma HDL-C does not always represent the level and function of HDL. The concentration of apoA-I is a more accurate measurement of HDL levels. Second, not all HDL is equal. It has been shown that HDL can be anti-inflammatory or pro-inflammatory (Navab et al., 2005). This may explain some of the discrepancies regarding the association of HDL levels with disease status in clinical studies. Thus, in addition to the quantity of HDL, a reliable and practical assay needs to be developed to measure the quality of HDL. Third, it is not clear if apoA-I has to be present in the brain to exert beneficial effects. Further studies are needed to dissect systemic and local effects of apoA-I/HDL on cognitive function. Lastly, the exact mechanism of action for HDL to modulate cognitive function has not been elucidated. Since HDL is a modifiable target, more studies are urgently needed in this regard. A small increase in functional HDL levels may have a profound capacity to prevent, delay and/or halt the progression of the diseases.

To address these gaps in the current literature, the present dissertation showed that CETP tg expression was able to modify the cholesterol profiles in mice. This modest

reduction of HDL-C levels in CETP tg mice did not affect the HDL's anti-oxidative properties as measured by PON1 activity. Additionally, CETP tg expressing had no significant effect on brain amyloid pathology in 6 (plaque formation onset) or 9 mo (plaque accumulation). Taken together, the modest changes to cholesterol profiles did not result in a significant change in cognitive function in AD mice co-expressing CETP. However, the present dissertation research showed that peripheral administration of an apoJ/clusterin mimetic, D-apoJ[113-122], robustly reduced brain amyloid plaque pathology by either increasing brain Aβ clearance or reducing brain Aβ reuptake from the plasma. Indeed, plasma amyloid levels were twice as high in D-apoJ[113-122]-treated AD mice. Additionally, D-apoJ[113-122] treatment dramatically reduced CAA, which could be a major mechanism involved in the improved behavioral outcomes in treated mice. Overall, these data suggest that apo/HDL mimetic peptides are a promising therapeutic agent for AD.

Protein prenylation is a critical lipid posttranslational modification of many important proteins. Particularly, it plays a key role in determining the cellular localization and functions of small GTPases. Small GTPases control signaling pathways that regulate a plethora of cellular functions including synaptic plasticity, and dysregulation or dysfunction of small GTPases leads to different types of disorders. Emerging evidence indicates that protein prenylation plays an important role in the development of AD. However, clinical trials using statins in patients with AD have not shown consistent benefits (Shepardson et al., 2011). While differences in the blood-brain barrier permeability and the dose of statins, the population of subjects, and the stage of the disease

at which statins are administered could all contribute to the discrepancies in clinical outcomes, one critical missing point is the fact that statins inhibit the production of FPP and GGPP simultaneously (Eckert et al., 2009) and thus may affect both farnesylation and geranylgeranylation pathways. Importantly, farnesylated and geranylgeranylated proteins are involved in regulating distinct cellular functions (Klooster 2007). Recent studies from our laboratory have demonstrated that specific inhibition of protein farnesylation but not geranylgeranylation enhances synaptic and cognitive function as well as reduces AD pathology (Cheng et al., 2013a), suggesting the potential of FTIs as therapeutic agents for AD. Findings from the present dissertation showed that inhibition of protein geranylgeranylation has negative effects on synaptic plasticity and dendritic spine density, further supporting the differential roles of two protein prenylation pathways in brain function and cautioning the use of general protein prenylation inhibitors for AD.

Our society is aging at an unprecedented pace, mainly due to longer life spans and the aging of the baby boomer generation. Aging itself remains the strongest risk factor for AD. Thus, the increasingly aged population will inevitably have a large impact on health care systems and national economies along with emotional and financial burden on the patients and their families. Consequently, therapeutic interventions aimed to increase the quality of life at advanced age are in high demand, both at the level of individuals and society. Safe and effective HDL-enhancing therapies may fulfil this demand. Additionally, further studies are needed to elucidate the role of protein prenylation, in particular farnesylation, on the onset and progression of AD. Until then, FTIs developed originally for the treatment of cancers may translate well to the treatment of AD.

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