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Apolipoprotein E receptor and cholesterol level alterations are pronounced in Alzheimer's Disease cortical synapses

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Apolipoprotein E receptor and cholesterol level alterations are pronounced in Alzheimer's Disease cortical synapses

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in Nursing

by

Bianca Gonzalez

2016

ABSTRACT OF THE DISSERTATION

Apolipoprotein E receptor and cholesterol level alterations are pronounced in Alzheimer's Disease cortical synapses

by

Bianca Gonzalez

Doctor of Philosophy in Nursing

University of California, Los Angeles, 2016

Professor Karen H. Gylys, Co-Chair

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Studies have identified amyloid beta (Aß) protein accumulation in the synapse as a neurotoxic hallmark associated with synaptic dysfunction, which occurs early in Alzheimer's Disease (AD) pathology. Clearance of soluble Aß from the synapse is thought to occur through binding of Aß to high density lipoprotein (HDL) and apolipoprotein E (apoE) complex in the brain. Studies suggest that the resulting Aß/apoE/HDL cholesterol complex is a mechanism of internalizing and clearing Aß from the synapse through apoE receptors, low density lipoprotein receptor (LDLR) and low density lipoprotein receptor related protein 1 (LRP1). This clearance process is thought to be dysfunctional in AD, however, evidence regarding specific molecular processes in the synaptic compartment involving apoE, Aß, LDLR, LRP1, and cholesterol across

AD stage and *APOE* genotype remains unclear. Therefore, levels of LDLR, LRP1, and cholesterol were measured in synaptosomes across AD stage and *APOE* genotype using flow cytometry and Western blot analysis.

Experimental results demonstrate that Aß and apoE levels were increased in synaptosomes that were postitive for LDLR and LRP1 in late AD cases, which demonstrates possible co-localization of apoE receptors with Aß and apoE. Additionally, LDLR was increased while LRP1 was reduced in late AD, suggesting that downregulation of LRP1 may contribute to dysfunction in Aß clearance while increases in LDLR in late AD may be associated with a compensatory clearance mechanism for processes that is inefficient. Furthermore, esterified cholesterol was reduced while free cholesterol was increased in late AD versus normals, suggesting that increased lipid storage may play a role in AD prevention and free cholesterol in late AD may be associated with membrane instability and subsequent synaptic loss. *APOE3/3* synaptosomes showed increased free cholesterol level and reduced esterified cholesterol level in late AD versus normals. *APOE+e4* synaptosomes, however, showed no signficant cholesterol level changes across AD disease stage, implying that dynamic cholesterol turnover is necessary for synaptic health. Overall, the results suggest that apoE receptors and cholesterol level alterations are associated with AD pathogenesis via disruption of Aß clearance mechanisms.

The dissertation of Bianca Gonzalez is approved.

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2016

DEDICATION PAGE

This dissertation is dedicated to my family for their multiple sacrifices and support. Without their continued encouragement, strength, and dedication to providing me with the best opportunities to advance my education, I would not have gotten to this point in my life.

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ABBREVIATIONS

24S-OH-C- 24S-hydroxycholesterol
3xTg-AD - mice contain three mutations associated with familial Alzheimer's disease (APP Swedish, MAPT P301L, and PSEN1 M146V)
α1-ACT - α1-antichymotrypsin
AAV8 - adeno-associated viral serotype 8
Aβ – amyloid β-protein
Aβ*56 – 56 kDa oligomer of amyloid ß-protein
Aβ40 − 40-residue isoform of amyloid β-protein
Aβ42 − 42-residue isoform of amyloid β-protein
ABCA1 - ATP-binding cassette transporter 1
ABCG1 - ATP-binding cassette transporter sub-family G member 1
ACAT - acyl-coA:cholesterol acyltransferase
AD – Alzheimer's Disease
ADDLs - Aß-derived diffusible ligands
Akt - protein kinase B
AMPA − 2-amino-3-(5-methyl-3-oxo-12- oxazol-4-yl) propanoic acid
ANOVA – analysis of variance
APOE – apolipoprotein E
ApoEr2 - ApoE receptor 2
APP – amyloid β-protein precursor
ATP – adenosine triphosphate
Arg – Arginine amino acid
ASO - miR-33 antisense oligonucleotide
BBB − blood-brain barrier
beclin1 – beclin 1 adaptor protein
C3 – complement component 3
CNS – central nervous system
CSF – cerebrospinal fluid
CYP46A1 - cholesterol 24-hydroxylase enzyme
Cys – cysteine amino acid
CREB - cyclic adenosine monophosphate responsive element-binding protein
DLG – discs-large
DSM - Diagnostic and Statistical Manual of Mental Disorders
ECE- endothelin-converting enzyme
eIF2 - Eukaryotic Initiation Factor 2
FAD – familial Alzheimer's disease
GAP-43 – growth associated protein 43
Glu – glutamic acid
GSK3ß - glycogen synthase kinase 3ß
HSPG - heparan sulphate proteoglycan
IL-1 – interleukin 1
LTP – long-term potentiation
LXR - liver x receptor
P38 MAPK - P38 mitogen-activated protein kinases

MAPT – microtubule association protein tau
MCI – mild cognitive impairment
MiRNA - microRNA
NEP - neprilysin
NFTs – neurofibrillary tangles
NMDA – N-methyl-D-aspartate
p-tau − hyperphosphorylated-tau
PD – Parkinson's disease
PI3 K - phosphoinositide-3-kinase
PICALM - phosphatidylinositol-binding clathrin assembly
PSD-95 – postsynaptic density protein 95
PSEN – presenilin
PPARγ - proliferator activated receptor gamma
RAGE - receptors for advanced end glycation products
RAP – receptor-associated protein
ROS – reactive oxygen species
RXRs - retinoid x receptors
SDS-PAGE – sodium dodecyl sulphatepolyacrylamide gel electrophoresis
SEM – standard error of the mean
SREBP1c - sterol regulatory element binding protein 1c
TNF-α - tumor necrosis factor-α
TNFR1 - tumor necrosis factor receptor 1
TRL – toll like receptors
Vps34 - Class III PI 3-kinase
VLDL - very low density lipoprotein receptor
WT – wild-type
Z0-1 - zona occludens 1

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Chapter 1

Introduction

1.1 Problem

Alzheimer's disease (AD) has a prevalence of 5.3 million in the United States and 35.6 million worldwide (World Health Organization, 2013; Centers for Disease Control and Prevention [CDC]). However, there is no definite treatment or curative therapy available to prevent the progression of the disease. Two types of AD include the familial and sporadic forms of AD. Familial AD (FAD) comprises approximately 5% of all AD cases, while 95% of all AD cases are sporadic (Alzheimer's Association, 2014). FAD has an earlier onset compared to sporadic AD and is attributed to mutations in the amyloid beta precursor protein (APP*)*, Presenilin 1 (PSEN1*)*, and Presenilin 2 (PSEN2*)* genes (Saunders et al., 1993; Guerreiro & Hardy, 2014). These mutations contribute to increased levels of amyloid beta (Aß) and phosphorylated tau (p-tau), which are pathological hallmarks in AD (Guerreiro & Hardy, 2014).

APOE genotype has been identified as the strongest genetic risk factor for developing the more prevalent sporadic form of AD (Leoni, 2011). The *APOE* gene transcribes apolipoprotein E protein (apoE), which is the main cholesterol transporter in the brain (Wildsmith, Holley, Savage, Skerrett & Landreth, 2013; Johnson et al., 2014). There are three alleles of apolipoprotein E (*APOE*) that have differing AD risk profiles: *APOE2* < *APOE3* < *APOE4* (Reitz & Mayeux, 2010; Leoni, 2011). Studies demonstrate that the apoE4 isoform is not as efficient in transporting cholesterol in the brain compared to isoforms apoE2 and apoE3 due to structural differences (Johnson et al., 2014; Frieden & Garai, 2013; Brodbeck et al., 2011).

Mounting evidence in animal and human *in vivo* and *in vitro* models shows a strong linkage between the *APOE e4* allele and increased Aß levels in the synapse (Johnson et al., 2014). Importantly, synaptic damage occurs early in AD and is associated with cognitive decline and increased soluble Aß levels in the synapse (Gylys et al., 2003). Therefore, multiple lines of evidence propose that lipidated apoE and Aß form a complex that is responsible for clearing Aß from the synaptic compartment (Gylys et al., 2003; LaDu et al., 1995; Tai et al., 2014; Garai, Verghese, Baban, Holtzman & Frieden, 2014). Additionally, the apoE/Aß complex is suggested to interact with cell surface apoE receptors for Aß internalization and degradation from the synapse (Gylys et al., 2003; Tai et al., 2014). This Aß clearance mechanism is thought to be dysfunctional in AD and contributes to neurotoxic Aß accumulation in the synapse (Gylys et al., 2007; Arold et al., 2012). Furthermore, because apoE is the main cholesterol transporter in the brain, dysfunctional cholesterol transport, cholesterol synthesis, and cholesterol metabolism are thought to contribute to AD pathogenesis (Djelti et al., 2015). However, molecular mechanisms in dysfunctional synaptic Aß clearance in relation to cholesterol dynamics across AD stage and *APOE* genotype are poorly understood.

1.2 Purpose of the Study

A growing body of literature suggests that dysfunctional Aß clearance in the synapse contributes to AD and may be related to cholesterol dynamics in the brain (Djelti et al., 2015; Vance, 2012; Gylys et al., 2007; Arold et al., 2012). However, it is unclear how AD pathology and *APOE* genotypical differences are associated with alterations in cholesterol constituents in the brain such as esterified cholesterol and unesterified cholesterol. Studies suggest that cholesterol-bound apoE forms a complex with soluble Aß that facilitates Aß internalization via apoE receptors for intracellular degradation (Gylys et al, 2003; Gylys et al., 2007; Arold et al.,

2012, Tai et al., 2014). Therefore, level differences in apoE receptors and cholesterol may contribute to Aß clearance dysfunction in AD. However, this remains unclear. Therefore, the purpose of this study is to explore associations of apoE receptor levels and cholesterol levels across AD stage and *APOE* genotype in cortical synapses. The overall hypothesis of this investigation is that dysfunctional synaptic Aß clearance in AD is a function of poor uptake from the downregulation of apoE receptors as well as altered cholesterol dynamics in the pathologic *APOE4* allele.

1.3 Specific Aims

1.3.1 Aim 1: ApoE receptor associations with apoE and Aß in AD synaptic pathology

Early experiments by Gylys and colleagues (2003) found that treating synaptosomes with soluble Aß and apoE increases Aß-positive synapses. Furthermore, when low density lipoprotein receptor related protein 1 (LRP1) inhibitor was applied to treated cells, Aß-positive synapses declined. This early evidence suggests that soluble Aß is cleared from the synapse via apoE transport to apoE receptors (Gylys et al., 2003). A study by Shibata et al. (2000) showed that apoE receptor knock-out mice had increased Aß accumulation and reduced clearance. This result further reinforces the hypothesis that apoE receptors are involved in Aß clearance. Therefore, this study aims to test this theory through measurement of Aß and apoE associated with low density lipoprotein receptor (LDLR) and LRP1 in AD synapses through dual-labeling Aß and apoE with apoE receptors. The working hypothesis is that Aß and apoE levels will increase in LDLR and LRP1-positive synapses, supporting the hypothesis that there is interaction between the Aß, apoE, and apoE receptors that may be involved in Aß clearance from the synapse.

1.3.2 Aim: 2 ApoE receptor associations with AD synaptic pathology

Because apoE is the primary transporter of cholesterol in the brain and is thought to function in Aß clearance through apoE receptor internalization, it is important to understand changes in apoE receptor levels in AD progression. ApoE is thought to carry Aß toward apoE receptors LDLR and LRP1 to transport Aß intracellularly for degradation (Zlokovic, 2013; Sagare et al., 2007). Additionally, multiple lines of evidence have shown associations between apoE isoform and apoE receptor levels in AD pathogenesis (Shu et al., 2014; Kim, Basak & Holtzman, 2009). However, differences in LDLR and LRP1 levels and function in Aß clearance from the synapse remain unclear. Therefore, this study aims to quantify LDLR and LRP1 in human synapses of normal versus early and late AD to determine associations between receptor levels and AD progression. The working hypothesis is that apoE receptors are responsible for Aß clearance in the synapse and therefore, LDLR and LRP1 will be downregulated in late AD compared to normal synapses, which may be why Aß clearance is impaired in AD.

1.3.3 Aim 3: Cholesterol levels and association with AD synaptic pathology

Cholesterol dynamics in the brain have been implicated in AD pathogenesis through membrane cholesterol alterations and lipid rafts (Murphy et al., 2013). Recent studies suggest that unesterified cholesterol (free cholesterol) that is located in the cell membrane facilitates signal transduction that is involved in learning and memory tasks (Murphy et al., 2013; Bryleva et al., 2010). Free cholesterol is converted to esterified cholesterol by acyl-coA:cholesterol acyltransferase (ACAT1:ACAT2) enzymes in human brain (Murphy et al., 2013). Esterified cholesterol forms intracellular lipid droplets, which are thought to function in lipid storage (Murphy et al., 2013; Bryleva et al., 2010). Additionally, esterified cholesterol is converted to

cholesterol 24S-hydroxycholesterol (24S-OH-C) by 24-hydroxylase enzyme (CYP46A1) (Murphy et al., 2013).

Recent evidence in AD transgenic mouse models shows that increased levels of esterified cholesterol are correlated with increased Aß aggregation and decline in cognitive function (Hutter-Paier et al., 2004; Bryleva et al., 2010). Furthermore, alterations of cholesterol levels through the manipulation of ACAT enzymes or 24-hydroxylase enzymes show that reduced esterified cholesterol and increased 24S-OH-C levels are associated with reduced Aß aggregation and improved cognitive function (Hutter-Paier et al., 2004; Bryleva et al., 2010). However, the impact of cholesterol dynamics on synaptic Aß clearance remains unclear. Therefore, this study aims to investigate associations in esterified cholesterol and free cholesterol levels in AD pathogenesis. Samples will be segmented by AD stage to reveal relevant cholesterol associations between normal versus early and late AD cases. The hypothesis is that esterified cholesterol and free cholesterol will trend in opposite directions across AD stage due to ACAT conversion of the cholesterol pools and that there exists an optimal cholesterol ratio for normal synaptic function.

1.3.4 Aim 4: Determine variations in esterified cholesterol and free cholesterol levels in relation to *APOE* genotype and AD stage

APOE genotypical variations are associated with differences in AD risk that may be related to apoE cholesterol-binding capacity. Therefore, it is important to investigate cholesterol levels in synapses across *APOE* genotype and AD stage (Liu et al., 2011; Dietschy, 2009; Murphy et al, 2013). Studies have shown that apoE and amyloid-β (Aβ) peptide binding may occur at residues 244–272 of apoE and residues 12–28 of Aβ, which varies according to apoE isoform (Liu et al., 2011; Sadowski et al., 2006). Furthermore, apoE is thought to mediate cholesterol processing through isoform-dependent LDLR binding. This suggests that apoE

isoform-dependent interactions with Aß that mediate Aß levels directly through the creation of an apoE/Aß complex (Tai et al., 2014; Verghese et al., 2013; Johnson et al., 2014). Therefore, this study aims to explore cholesterol level differences in across *APOE* and AD stage by measuring esterified cholesterol and free cholesterol in synaptosomes across *APOE* and AD stage.

Chapter 2

Review of the Literature

2.1 Alzheimer's Disease

2.1.1 Background

Alzheimer's Disease (AD) is the most common form of dementia and is characterized by progressive decline of multiple cognitive functions. The Diagnostic and Statistical Manual of Mental Disorders (DSM) Fifth Edition published by the American Psychiatric Association defines dementia as a neurocognitive disorder with severe symptoms that interfere with activities of daily living. Criteria include deficits in at least one of the following: 1) ability to comprehend language and speak coherently; 2) ability to perform voluntary motor activities; 3) ability to recognize and identify objects and 4) ability to plan and carry out complex tasks and make decisions (American Psychiatric Association, 2013).

AD is the sixth leading cause of death in the U.S. and the leading cause of dementia worldwide (Alzheimer's Association, 2014). The prevalence of AD is expected to double by 2030 and triple by 2050. The annual increase in incidence from 2010 to 2050 is estimated to be 16.15 million due to an increase in the aging population (World Health Organization, 2012).

The cost to treat AD places a significant economic burden on societies worldwide. In the United States, costs related to the treatment of AD are approximately $172 billion per year. Worldwide, the cost of AD is approximately $604 billion per year (World Health Organization, 2013; Centers for Disease Control and Prevention [CDC], 2013). These cost estimates include the cost of providing health care, public programs, and the loss of income of people with AD and their caregivers. Additionally, AD is associated with twice more hospital admissions than those without AD. Bacterial pneumonia, congestive heart failure, dehydration, duodenal ulcer, and

urinary tract infection are among the most prevalent conditions for which AD patients are hospitalized (Phelan, Borson, Grothaus, Balch & Larson, 2012). Moreover, while the World Health Organization has classified AD as a priority disease, AD does not have a definite treatment, cure, or prevention (Alzheimer's Association, 2014; World Health Organization, 2013).

2.1.2 Risk Factors

The most significant risk factor for developing AD is age. AD risk increases exponentially with increasing age and is most prevalent among people over the age of 65. The risk for developing AD reaches 50% for individuals older than 85 years of age (World Health Organization, 2013).

In addition to age, genetic factors contributing to AD have been identified. In the early 1990s, linkage analyses studies identified that fully penetrant mutations in the *APP, PSEN1*, and *PSEN2* genes are involved in early onset AD (less than 60 years of age). Additionally, the *e4* allele of *APOE* was identified as a strong risk factor for late-onset familial and sporadic AD through family studies (Saunders et al., 1993; Guerreiro & Hardy, 2014). More recently, over 20 genetic loci have been identified by Genome-Wide Association Studies (GWAS) that examined common genetic variants of AD between large groups of cases and controls. In terms of biological pathways, there are three main pathways that are associated with AD pathology, suggesting that these processes may pose significant risk factors leading to AD. These processes include alterations in cholesterol and lipid metabolism, endosomal vesicle recycling, and immune response activation (Guerreiro & Hardy, 2014).

2.1.3 Clinical Progression

While the most prominent clinical feature of AD is memory loss, progression of AD involves the overall decline of cognitive function (Ewers et al., 2012). Mild cognitive impairment (MCI) has been recognized as an early clinical sign of future progression to AD

(Douaud et al., 2013). However, not all MCI patients progress to AD, suggesting that there are multiple etiologies of MCI that do not pertain to underlying AD pathology (Douaud et al., 2013; Ewers et al., 2012). Therefore, it is difficult to identify AD during its early stages through clinical symptoms alone.

Based on a systematic review and analysis of the literature from the National Alzheimer's Coordinating Center (NACC) database, the National Institute of Aging (2013) consensus defines stages in AD clinical progression. These include preclinical, mild cognitive impairment, and AD, where AD refers to the presence of pathological changes observed at postmortem autopsy (Hyman et al., 2013). To enhance the detection and diagnosis of AD during its early stages, biomarker based quantification has been used in diagnostic testing of early AD progression. Two pathological hallmarks of AD include neurofibrillary tangles (NFTs) that are composed of filamentous tau protein and neuritic Aß plaques (Hyman et al., 2013; Ewers et al., 2012; Mattsson et al., 2009; Nelson, Kukull & Frosch, 2010).

2.1.4 Pathophysiology

Research in animal models, neuronal cell cultures, and human postmortem tissue show that AD pathogenesis is a function of increased Aß peptide aggregation and reduced Aß clearance in the brain (Ye et al., 2005). These processes contribute to neurotoxicity and neuronal cell death (Bayer and Wirths, 2010). Because of the mounting evidence of the role of Aß in AD pathogenesis, many studies have investigated the dynamics of Aß aggregation and clearance mechanisms.

In the monomeric form, Aß is a peptide that is approximately 4.5kDa and 39- to 42-amino acid residues long. It is capable of forming plaque aggregates, which are associated with

AD pathogenesis (Nag et al., 2011). Aß is produced by ß-secretase and subsequent γ-secretase cleavage of amyloid precursor protein (APP) (Hardy and Higgins, 1992; Chemuru, Kodali, and Wetzel, 2015). The variation in length of Aß species is determined by processing through the γ-secretase pathway (Ahmed et al., 2010; Chemuru, Kodali, and Wetzel, 2015).

Aß40 and Aß42 peptides are the two main conformations of Aß in the human brain. Aß42 is more prone to aggregation and formation of neurotoxic fibrils than Aß40 (Chemuru, Kodali, and Wetzel, 2015). Nuclear magnetic resonance spectroscopy (NMR) demonstrates that Aß42 fibrils are 42-residues in length and comprised of ß-strands that are arranged in a cross-ß structure. The N-terminal of Aß42 is unstructured while the hydrophobic ß-strand regions within the fibril portion of Aß42 have a parallel orientation (Ahmed et al., 2010). Of particular interest are residues 14−23 of Aβ due to involvement in fibril formation. Residues 14-23 of Aβ are capable of self-recognition and subsequent self-binding. This property enables Aß to bind to other Aβ peptides facilitating aggregation and plaque formation (Takahashi and Mihara, 2008).

Pertaining to neurotoxicity, Aß monomers are capable of aggregating and producing intermediates such as soluble Aß oligomers and insoluble Aß fibrils (Ahmed et al., 2010; Takahashi and Mihara, 2008). Aß fibrils aggregate and form benign amyloid plaques while soluble oligomeric Aß directly causes neurotoxicity and neuronal cell death through post-synaptic interactions with glutamate receptors, cellular prion protein, neuroligin, Wnt, and insulin receptors (Ferreira, Lourenco, Oliveira and Felice, 2015; Ferreira and Klein, 2011; Oliveira and Felice, 2015). Furthermore, studies show that Aß oligomers interact with receptors pertinent to learning and memory such as AMPA, NMDA, and type 5 metabotropic glutamate receptors (mGluR5) (Ferreira, Lourenco, Oliveira and Felice, 2015; Zhao et al., 2010; Um et al., 2013). These interactions are implicated in memory loss and cognitive dysfunction in AD.

Evidence from immunofluorescence experiments shows that Aß oligomers localize in glutamatergic, excitatory synapses as well as axons and dendrites (Sokolow et al., 2012; Ferreira, Lourenco, Oliveira and Felice, 2015; Baleriola et al., 2014). Sokolow and colleagues (2012) investigated glutamatergic terminal loss in AD and demonstrated that high levels of Aß localize in presynaptic glutamatergic terminals in AD. This evidence points to Aß-mediated glutamate dysfunction playing a role in AD pathogenesis (Sokolow et al., 2012). Furthermore, Aß oligomer binding to axons and synapses is thought to inhibit efficient synaptic transmission and axonal transport. In terms of molecular mechanisms, it has been demonstrated that Aß oligomers trigger an inflammatory response that increases levels of tumor necrosis factor-α (TNF-α) and tumor necrosis factor receptor 1 (TNFR1) activity (Lourenco et al., 2013). This is thought to increase eukaryotic initiation factor 2 (eIF2α) phosphorylation, which is correlated to synaptic loss in AD and impaired learning and memory (Lourenco et al., 2013; Ma et al., 2013; Yoon et al., 2012).

2.1.5 Plaque Progression and Staging

Amyloid plaque aggregation occurs before the onset of overt cognitive dysfunction and follows a prominent pattern of growth during the early stages of AD. Amyloid plaques are composed of aggregated Aß peptide found in the extracellular space of neuronal tissue (Yan et al., 2009; Meyer-Luehmann et al., 2008; Bolmont et al., 2008). Yan et al. (2008) found that the rate of plaque growth is associated with initial plaque size where small Aß aggregates showed greater rates of growth compared to larger ones independent of age (Yan et al., 2008).

The preferred method for Aβ plaque detection is immunohistochemistry for Aβ. Other methods include Thioflavin-S or silver histochemical stains (Hyman et al., 2013). The Consortium to Establish a Registry for Alzheimer's disease (CERAD) protocol recommends Thioflavin-S or modified Bielschowsky for Aß plaque detection (Mirra et al., 1991). Amyloid

plaques are classified based on morphology and positive or negative staining with Thoflavin-S or Congo Red. These classifications include: 1) diffuse and 2) dense-core plaques. Dense-core plaques are fibrillar amyloid deposits with compact core that stains with Thioflavin-S and Congo Red. They are commonly surrounded by neuritic plaques, reactive astrocytes, and activated microglial cells. These plaques are associated with synaptic loss, neuronal degeneration, and cognitive impairment used for the diagnosis of AD. In contrast, diffuse plaques are composed of Aß aggregations with irregular borders that are not stained by Congo Red and Thioflavin-S. Diffuse plaques are not usually correlated with glial activation or synaptic degradation. Therefore, the diffuse plaque type is not considered a pathological hallmark of AD and has been a common finding in cognitively normal elderly people (Serrano-Pozo, Frosch, Masliah & Hyman, 2011).

Early in AD, Aß oligomers are soluble and are able to diffuse from gray matter to white matter. Aggregation of insoluble Aß is seen in later stages of disease progression. In Braak stage I, Aß is mainly located in the magnocellular nuclei of the basal forebrain and the locus ceruleus (Busch, Bohl & Ohm, 1997; Braak & Del Tredici, 2004). In later stages of AD progression, Aß deposits appear in the striatum and hippocampus (Thal et al., 2000; Braak & Del Tredici, 2004).

The National Institute of Aging classifies AD neuropathologic change according to three parameters to calculate an overall "ABC" score. The "ABC" score is comprised of: 1) Aß plaque score, 2) Braak NFT stage, 3) CERAD neuritic plaque score. The Aß plaque score is based modified criteria from Thal et al. (2009). During the A0 stage there are no amyloid plaques, the A1 stage is characterized by sparse neuritic plaques and low NFT density with neocortical involvement, the A2 stage exhibits Aß progression into the hippocampus, diencephalic nuclei,

the striatum, and the cholinergic nuclei of the basal forebrain, and the final A3 stage is classified by cerebellar Aß deposits and involvement in the neostriatal regions. Overall, Aß deposition follows a general sequence progressing in an anterograde direction (Hyman et al., 2013; Thal, Rüb, Orantes & Braak, 2002). Neuritic plaque score is derived from the CERAD criteria where the C0 phase shows no neuritic plaques, the C1 stage is characterized by sparse neuritic plaques, C2 phase shows moderate neuritic plaques, and C3 phase shows frequent neuritic plaques (Mirra et al., 1991). The summative Aß, neuritic plaque, and NFT score contribute to the final "ABC" score (Hyman et al., 2013).

2.1.6 Tangle Progression and Staging

Like amyloid plaque accumulation, NFT aggregation is also associated with AD pathology and is parallel to the onset and severity of cognitive deficits. Likewise, neuronal and synaptic degeneration follow a pattern of NFT progression. However, it remains undetermined whether or not tangles cause neuronal degeneration (Serrano-Pozo, Frosch, Masliah & Hyman, 2011).

NFTs are composed of intraneuronal fibrils of tau protein. NFTs can be quantified and visualized with histochemical stains or with immunohistochemistry directed against tau or phospho-tau epitopes. Braak and colleagues described the neuropathological stages through the AD continuum explaining neuronal destruction in differing brain regions. There are six stages in AD progression, Braak Stages I-VI. According the Braak hypothesis, NFTs innervate neurons beginning in the entorhinal cortex, and over time, spreads to the hippocampus and limbic system. Later, neurodegeneration spreads to the primary motor cortex, temporal lobe, and occipital lobe (Braak, Alafuzoff, Arzberger, Kretzschmar, & Del Tredeci, 2006). Cognitive decline and symptomatology of AD is associated with stage IV-VI limbic degeneration involving

dysfunction in multiple neurotransmitters such as dopamine and cholinergic systems (Braak, Alafuzoff, Arzberger, Kretzschmar, & Del Tredeci, 2006).

In the earliest stage of AD, Braak stage I there are no NFTs. In Braak stages I-II, NFTs are seen mainly in entorhinal cortex and is concentrated in both the cell body and neuronal processes of neurons in the subcortical nuclei of the transentorhinal region (Braak, Alafuzoff, Arzberger, Kretzschmar, & Del Tredeci, 2006). In later phases of Braak stage II, these pathological lesions spread towards the temporal neocortex. In Braak stage III, lesion density is increased and extends into the fusiform gyri, lingual gyri, and is more abundant in hippocampus and amygdala (Hyman et al., 2013). Additionally, NFTs extend into association cortex. Braak stage IV lesions progress towards high order sensory association areas of the temporal lobe and into deeper cortical regions of CA3 and CA4 involving mossy cell neurons. In Braak stage V, pathological tau lesions extend in a fan-like pattern towards frontal, superolateral, occipital lobes, and higher order association areas and involve some pyramidal cells in deeper layers III-V (Braak, Alafuzoff, Arzberger, Kretzschmar, & Del Tredeci, 2006). In Braak stage VI, pathological lesions reach the occipital lobe extending into the striatal area. Most areas of the cortex show severe tau lesion invasion extending to almost all layers (Braak, Alafuzoff, Arzberger, Kretzschmar, & Del Tredeci, 2006; Hyman et al., 2013).

2.1.7 Intraneuronal Pathology and Loss

Aß accumulation has traditionally been thought of as an extracellular process due to the high prevalence of Aß plaque aggregation extracellularly. However, mounting evidence shows that Aß accumulation occurs intracellularly (Capetillo-Zarate, Gracia, Tampellini & Gouras, 2011). Several labs have demonstrated that intracellular Aß accumulation occurs before extracellular Aß plaque formation (Gouras et al., 2000; Gouras, Tampellini, Takahashi, &

Capetillo-Zarate, 2010; Oakley et al., 2006). In terms of the relationship between intracellular and extracellular Aß, Oddo and colleagues (2006) found that there are inverse levels of intraneuronal Aß and extracellular Aß plaque, suggesting movement of Aß from one compartment to another. Additionally, correlations exist between intraneuronal Aß decreases and age, while Aß extracellularly increases with age in 3xTg-AD mice **(**Oddo, Caccamo, Smith, Green & LaFerla, 2006).

Utilizing immunohistochemistry and digital image analysis, D'Andrea and colleagues (2001) found that Aß selectively accumulates in cathepsin D-positive granules, which suggests lysosomal structural involvement with Aß. More importantly, investigators found neurons that were heavily burdened with Aß42 underwent neuronal lysis, evidenced by the dispersion of Aß42 and lysosomal enzymes in the extracellular space. D'Andrea and colleagues (2001) also found that there were inverse levels of neuronal density and amyloid plaque density in AD (D'Andrea et al., 2001). This evidence supports experiments in transgenic mouse models that have found positive correlations between neuronal cell death and intraneuronal Aß accumulation (Bayer and Wirths, 2010; Oakley et al., 2006).

2.1.8 Synapse Loss

In healthy individuals who do not have cognitive deficits, synaptic plasticity in the brain facilitates learning and memory formation, allocation, and consolidation through synaptic receptor trafficking and transmission (Rumpel, LeDoux, Zador & Malinow, 2005). In AD, severe memory deficits are manifested, which can be attributed to alterations in synaptic plasticity. Long-term potentiation (LTP) is a long-term increase in synaptic transmission between neurons

that are activated simultaneously. LTP has been associated with the strengthening of synaptic connections and the facilitation of learning and memory. Initial mechanisms in LTP involve kinase activation causing phosphorylation and upregulation in postsynaptic AMPA receptors (Won & Silva, 2008). Continued activation of kinases at postsynaptic sites accompanied with increased calcium leads to activation of cyclic adenosine monophosphate responsive element-binding protein (CREB). CREB has been associated with regulation of synaptic potentiation and long-lasting forms of memory (Won & Silva, 2008; Zhou et al., 2009; Spires-Jones & Hyman, 2014). Conversely, long-term depression (LTD) is a weakening of synaptic transmission following a stimulus and is thought to be associated with clearing old memory traces (Collingridge et al., 2010; Massey & Bashir, 2007; Spires-Jones & Hyman, 2014).

Through experiments involving the introduction of NFTs and oligomeric Aß in healthy rodents in vivo, LTP is disrupted by oligomeric Aß, which is demonstrated by marked decline in learning and memory (Shankar et al., 2008; Walsh et al., 2005). Moreover, Aß and tau co-localize in the synaptic compartment and have been implicated as contributors to synaptic loss in AD (Fein et al., 2008; Serrano-Pozo, Frosch, Masliah & Hyman, 2011; Crimins, Pooler, Polydoro, Luebke & Spires-Jones, 2013). Therefore, tau and Aß aggregation in the synapse is associated with overall learning and memory impairment (Spires-Jones & Hyman, 2014; Crimins, Pooler, Polydoro, Luebke & Spires-Jones, 2013).

From immunohistochemical studies using electron microscopy and synaptophysin antibody against synaptic proteins, it has been shown that synaptic loss follows a similar spatial pattern to neuronal loss and can even surpass neuronal loss in particular cortical areas in AD (Serrano-Pozo, Frosch, Masliah & Hyman, 2011). Evidence from cultured neurons and postmortem neuronal tissue show that the earliest sites of Aß accumulation and abnormal tau

phosphorylation occur in the synaptic compartment (Gouras, Willén & Faideau, 2014). Alterations in synapses are associated with cognitive impairment and it has been shown that synaptic activity regulates Aß secretion (Tampellini & Gouras, 2010; Gouras, Willén & Faideau, 2014). In studies that examined hippocampal slices of mouse neuronal tissue, increased synaptic activity facilitates Aß secretion while synaptic inhibition decreases Aß secretion (Tampellini & Gouras, 2010; Kamenetz et al., 2003; Cirrito et al., 2008). This mechanism of increased Aß secretion in the extracellular space leads to Aß aggregation, decreased synaptic plasticity, and loss of synapses (Tampellini & Gouras, 2010; Shankar et al., 2008; Deshpande et al., 2009).

2.1.9 Animal Models of Alzheimer's disease

The discovery of FAD mutations has prompted the development and creation of animal models expressing mutated *APP, PSEN1,* and *PSEN2* genes in AD that increase Aß production. Currently, 30 APP mutations and approximately 200 *PSEN1*/*PSEN2* mutations have been identified (Cruts, Theuns & Van Broeckhoven, 2012). Although familial Alzheimer's disease (FAD) mutations account for less than 5% of all AD cases, they are valuable due to the high degree of phenotypical similarity to sporadic AD (Selkow, 2001; Puzzo, Lee, Palmeri, Calabrese & Arancio, 2014). These models assume that there are similar underlying mechanisms in FAD and in sporadic AD to detect progression of AD (Puzzo, Lee, Palmeri, Calabrese & Arancio, 2014).

2.1.9.1 Transgenic and non-transgenic mouse models

Transgenic mouse models of FAD have been used widely to investigate AD progression and therapeutic targets. There are several lines of transgenic mouse models with mutations in *APP, PSEN1* and *PSEN2* genes. For instance, *APP* transgenic mutants overexpress human APP and this leads to increased Aß plaque aggregation and cognitive deficits (Puzzo, Lee, Palmeri,

Calabrese & Arancio, 2014). Co-mutation models that express alterations in *APP* and *PSEN1* lead to early onset AD and increased Aß (Holcomb et al., 1998; Puzzo, Lee, Palmeri, Calabrese & Arancio, 2014). When mutations in *APP* and *PSEN1* are combined with a mutation in *MAPT* (which is associated with frontotemporal dementia), such animals also demonstrate tangle development and significant neuronal death (Oddo et al., 2003).

To model the development and pathogenesis of sporadic AD, non-transgenic animal models of AD are also utilized to elucidate mechanisms in AD progression. These non-transgenic models are beneficial because the overexpression of *APP* and *PSEN1* in transgenic models may impact cognitive function utilizing mechanisms independent of Aß pathology (Kamal et al., 2001; Puzzo, Lee, Palmeri, Calabrese & Arancio, 2014). Other benefits of using non-transgenic animal models are cost reduction and the possibility to use animals other than mice, such as rats and non-human primates (Asle-Rousta et al., 2013). These models involve the intracerebroventricular injections of Aß, tau, or observation of aged animals (Puzzo, Lee, Palmeri, Calabrese & Arancio, 2014). Both transgenic and non-transgenic models have aided in discoveries in AD pathological mechanisms and facilitated the development and evaluation of novel therapeutics.

Transgenic Mouse Models			
Model/Mutation	**Description**	**Characteristics**	**Example**
APP (Swedish)	ß-secretase cleavage site mutation on amino acid 670/2	• Increased APP cleavage by ß-secretase • Increased Aß plaque production at 9-	Tg2576

		12 months • Tau hyperphosphorylation • Synaptic deficits • Memory deficits	
APP (Indiana, London)	Gamma-secretase cleavage site mutation on amino acid 717)	• Increased APP cleavage by gamma-secretase • Increased Aβ 42:40 ratio • Aß plaque aggregation at 9-12months • Tau hyperphosphorylation • Synaptic deficits • Cognitive deficits	PDAPP (Indiana)
APP (Dutch, Flemish, Italian, Arctic)	Aβ peptide mutation on amino acid 692/3	• Increased Aβ oligomerization at 9-12 months • Formation of protofibrils • Tau hyperphosphorylation • Synaptic deficits • Cognitive deficits	TgAPParc, APPDutch
APP (Japanese)	Aβ peptide	• Increased Aβ	

	mutation on amino acid E693delta Deletion of glutamate 22	oligomerization • No plaque formation • No fibrilization • Intracellular accumulation of Aβ oligomers at 8 months • Tau hyperphosphorylatoin • Neuron loss over 24 months • Synaptic deficits • Cognitive deficits	TgAPP (E693)
Combined APP mutations	Combination of single APP FAD mutations	• Increased Aß • Synaptic deficits • Cognitive deficits	TgCRND8, J20
Amyloid-β (Aβ)	Human Aβ40 or Aß 42 fusion protein with BRI	• Overexpression and secretion of only Aβ • No extra APP cleavage products • Reactive gliosis and amyloid pathology only with Aβ42 model	BRI-Aβ42

		• Synaptic deficits • Cognitive deficits	
Presenilin-1	FAD point mutations or exon 9 deletion in human PS1	• Increased Aβ 42:40 ratio • No plaque formation • Accelerated neurodegeneration in older mice >13mo • Synaptic deficits • Cognitive deficits	PS1 (M146V/L), PS1(dE9)
Tau	Point mutations in human MAPT (FTD mutations)	• Increased tau phosphorylation • Increased tau aggregation • No plaque formation • Motor defects • Synaptic deficits • Cognitive deficits	JNPL3, MAPT (P301L), MAPT(VLW)
APP/PS1	Double transgenic (APP FAD mutant overexpression, PS FAD	• • Early onset of cognitive deficits • Aß plaque formation early, 3-6 months	APP(swe)/PS1(M146L), APP(swe)/PS1(A246E)

	mutant expression or knock-in)	• Significant hippocampal neuron loss • Cognitive deficits • Synaptic deficits	
APP/Tau	Double transgenic (APP FAD mutant overexpression, tau FTD mutant overexpression)	• Early cognitive deficits • Minimal neurodegeneration • Aß plaques at 9 months • Synaptic deficits • Cognitive deficits	APP(swe)/tau (P301L), APP (swe)/tau (VLW)
APP/PS1/Tau	Triple transgenic; FAD APP and FTD tau transgenes in PS1 FAD knockin	• Increased Aß plaque aggregation at 3-6 months • Early Aß intraneuronal deposits • Plaques precede tangles • Synaptic deficits • Cognitive deficits	3xTgAPP [APP(swe)/PS1(M146V)/MAPT(P301L)]
Non-Transgenic Mouse Models			

Aβ injection	Direct infusion of Aβ into the brain	• Aβ elevation • Synaptic deficits • Cognitive deficits	Cell-derived Aβ, Aβ dimers from human brain, 200 nM synthetic Aβ oligomers
Tau injection	Direct infusion of tau into the brain	• Tau levels increased • Synaptic deficits • Cognitive deficits	Tau oligomers from human brain, recombinant tau oligomers
Aged animal models	Aged mice, rats, dogs, non-human primates	• Age-related cognitive deficits • Exhibit cognitive deficits • Cholinergic deficits • Altered calcium homeostasis • Oxidative stress • Synaptic deficits	Aged mice (>18–20 months old)

(Adapted from Puzzo, Lee, Palmeri, Calabrese & Arancio, 2014)

2.1.9.2 Non-human primate models

Due to structural and functional differences between mouse and human brain, non-human primate models of AD have been developed. Advantages of using primates in studying the progression of AD include high biologic similarity to humans, large brain mass, similar complex behavior to humans, and the ability to generate Aβ plaques during aging (Jebelli & Piers, 2015; Heuer, Rosen, Cintron & Walker, 2012). Furthermore, non-human primates do not develop AD

presentation in aging and may be useful in elucidating Aß dynamics in absence of neurodegeneration (Jucker, 2010). However, high cost and differences in primate pathological expression of AD compared to human AD has limited their use.

2.1.9.3 Limitations of animal models

Although models of AD have provided valuable insight into the progression of AD by reproducing pathological lesions in human AD, there are several limitations to their use due to differences of animal Aß compared to human Aß. In terms of localization, neuronal death in transgenic mice is located primarily in the hippocampus and differs from ubiquitous localization in human AD (Jucker, 2010; Duyckaerts, Poiter & Delatour, 2008). Biochemically, positron-emission tomography (PET) imaging studies of Pittsburgh Compound B (PIB) ([^{11}C]6-OH-BTA-1), which is a PET radiotracer that is capable of labeling individual Aß plaques, demonstrate stronger PIB binding to human Aß compared to Aß in mice (Klunk et al., 2005). Similar binding patterns have been demonstrated in non-human primates (Jucker, 2010; Rosen, Walker & Levine, 2009). In terms of structure, Lv and colleagues (2013) utilized circular dichronism spectroscopy, fluorescence assays, and transmission electron microscopy (TEM) and found that there are three amino acid variations in mouse Aß42 that differ from human Aß42. Results from this study also showed that mouse because of these mutations, mouse Aß42 was less prone to form ß-sheet structures, less prone to aggregation, less neurotoxicity, and less reactive oxygen species (ROS) production compared to human Aß42 (Lv et al., 2013). This evidence suggests that there are significant differences in localization, structure, and function between animal Aß and human Aß, limiting the use of animal models in the interpretation of efficacy of novel therapeutics. In fact, several novel therapies that demonstrated efficacy in reversing or slowing the progression of AD pathology in animal models had failed in treating AD in human brain (Cummings, Morstorf &

Zhong, 2014). Therefore, models that resemble human AD pathology must be developed to elucidate the intricacies of human AD pathogenesis.

2.1.10 Amyloid-ß Localization

Evidence shows that Aß accumulates in brain and cerebrospinal fluid (CSF) in AD (Herskovits et al., 2013; Gong et al., 2003). Aß concentrations in the brain and CSF have been implicated in AD progression and are associated with cognitive decline (Blennow et al., 2015; Bjorklund et al., 2012). Because multiple medications for AD have failed to show significant effect on pathological disease state, research on Aß localization in brain and CSF may produce insights into development of biomarkers for monitoring disease progression and efficacy of novel therapies (Blennow et al., 2015; Cummings, Morstorf & Zhong, 2014). Therefore, understanding the dynamics of Aß localization in the brain and CSF is imperative in monitoring progression of AD.

2.1.10.1 Neuronal Localization

In terms of neuronal localization, immunofluorescence and cell culture studies demonstrate that Aß oligomers accumulate in excitatory synapses and alter structural and functional properties (Takahashi et al., 2004; Kokubo et al., 2005). Additionally, Aß oligomers accumulate in synapses of AD brain when cognition is compromised (Bjorklund et al., 2012; Perez-Nievas et al, 2013; Forny-Germano et al., 2014). However, Aß oligomers are not seen in synapses when there is absence of cognitive dysfunction. Interestingly, it has been demonstrated that cognitively normal brain can contain Aß oligomers outside of the synapse. Neuropathological studies show that some individuals have large amounts of intraneuronal Aß without experiencing dementia (Bjorklund et al., 2012; Riley, Snowdon, Desrosiers, &

Markesbery, 2005). This evidence suggests that Aß oligomers are a precursor of cognitive decline and may be responsible for cognitive deficits in AD (Ferreira, Lourenco, Oliveira & Felice, 2015; Velasco, 2012). Moreover, in studies with APP transgenic mice where *de novo* production of Aß was inhibited, synaptic loss and memory impairment was reversed (Fowler et al., 2014).

Imaging technology such as magnetic structural resonance imaging (MRI) has influenced AD diagnosis in the clinical setting. Hippocampal and whole brain atrophy are considered markers of neurodegeneration, while atrophy of the medial temporal lobe is a valid diagnostic of MCI staging (Frisoni, Fox, Jack, Scheltens & Thompson, 2010). Furthermore, recent developments in Aß imaging agents for positron emission tomography (PET) have been clinically implemented as diagnostic agents to quantify Aß density in AD and dementia. These include highly sensitive and specific radioactive Florbetaben agents: ^{18}F-florbetapir, ^{18}F-flutemetamol, and ^{18}F-florbetaben (Maya et al., 2015; Barthel et al., 2011). Florbetaben agents bind to β-amyloid plaques in AD brain sections and had been validated by radiography, immunohistochemistry and Bielschowsky staining. Florbetaben is highly specific for Aß deposits and does not bind to tau or α-synuclein (Sabri, Seibyl, Rowe & Barthel, 2015). In a study that used Florbetaben tracer with PET imaging to study mice that overexpress APP (Swedish mutation), Rominger and colleagues (2013) observed temporal and spatial progression of Aß pathology. Researches found that Florbetaben binding progressed frontally and parietally with increasing age. Aged mice demonstrated fibrillar Aß plaques in frontal cortex and small amounts in hippocampus. Histochemical quantification was used to validate the results of the Florbetaben tracer (Rominger et al., 2013). The tracer is now approved by the U.S. Food and Drug

Administration and European Medicines Agency and had successfully completed global multicenter phase 0–III trials (Sabri, Seibyl, Rowe & Barthel, 2015).

2.1.10.2 CSF Localization

In addition to neuronal Aß localization, much focus has been drawn to Aß localization in CSF due to its potential utilization as a diagnostic marker for progression of AD in clinical trials and clinical settings. Studies have shown that Aß is can be eliminated from the brain through perivascular and interstitial fluid pathways into the CSF (Ueno, Chiba, Matsumoto, Nakagawa & Miyanaka, 2014). Additionally, it has been observed that Aß42 is decreased in CSF of AD patients compared to cognitively normal controls (Andreasen et al., 2001; Riemenschneider et al., 2002). Furthermore, studies have found that an inverse relationship exists between CSF Aß levels and cortical Aß levels (Strozyk, Blennow, White & Launer LJ, 2003; Tapiola et al., 2009). This evidence suggests that Aß in CSF is a potential mechanism for cortical Aß clearance. Therefore, dysfunctional in CSF clearance pathways can reduce Aß level in the CSF, reflecting inverse associations to cortical AD pathology (Blennow et al., 2015).

Due to the mounting evidence of Aß localization in CSF, new clinical guidelines by the European Federation of Neurological Societies (EFNS) recommend CSF analysis in the differential diagnosis of AD (Hort et al., 2010). However, in the U.S., the National Institute on Aging and the Alzheimer's Association (NIA-AA) do not currently recommend routine CSF testing for AD detection due to lack of research, validation, and standardization (Dubois et al., 2007; McKhann et al., 2011; Blennow et al., 2015). There is a need for further investigation on the utilization of CSF to detect Aß to demonstrate clinical efficacy and feasibility.

2.1.11 Amyloid Cascade Hypothesis and Elaborations

The initial observations by Alois Alzheimer in 1907 of senile plaques and NFTs in dementia led to the identification of Aß, which is the main constituent of the observed senile plaques (Alzheimer, 1987). Thereafter, familial studies discovered that mutations in *APP, PSEN1* and *PSEN2* genes caused increased Aß aggregation and early onset FAD (Goate et al., 1991; Levy-Lahad et al., 1995; Sherrington et al., 1995). This evidence led to the formulation of the Amyloid Cascade Hypothesis by Hardy and Higgens in 1992.

According to the amyloid cascade hypothesis proposed by Hardy and Higgens (1992), Aß peptide originates from proteolytic cleavage of amyloid precursor protein (APP) by ß-secretase and γ-secretase enzymes (Hardy & Higgins, 1992; Hardy 1997). In AD, the pathologic form of Aß is the Aß1-42 isoform, which is produced from the misfolding and cleavage of APP. Overproduction of Aß1-42 peptide or inefficient clearance of Aß1-42 peptide is thought to create aggregate stress from accumulation of plaques that block neuronal transmission, leading to synaptic and neuronal toxicity (Shinohara, Petersen, Dickson & Bu, 2013; Thinakaran & Koo, 2008; Reitz, 2012)

To add to the amyloid cascade hypothesis, studies have shown that Aß is cleared and degraded through uptake by intracellular lysosomes, glial cells, and drainage into the cerebrospinal fluid (Ueno, Chiba, Matsumoto, Nakagawa & Miyanaka, 2014). The mechanism of intracellular Aß clearance through lysosomal autophagy is mediated by apoE and lipoprotein cell surface receptors (Shinohara, Petersen, Dickson & Bu, 2013; Jiang, et al., 2008).

Although seemingly cogent, the amyloid cascade hypothesis is widely disputed. One argument against it is Aß aggregation and NFTs may not be the cause of neurodegeneration in AD, but rather, a result of neurodegeneration (Reitz, 2012). For example, in human head trauma studies, increased expression of APP and Aß deposits have been observed implicating Aß

deposition as a reactive process towards neuronal injury (Ikonomovic et al., 2004; Gentleman, Nash, Sweeting, Graham & Roberts, 1993; Roberts et al., 1994). Because of this, some investigators have proposed that high levels of APP observed in AD are a result of neuronal injury for the purposes of neuronal repair and survival (Regland & Gottfries, 1992; Reitz, 2012).

2.1.12 Oligomeric Aß

2.1.12.1 ADDLs, Protofibrils, and Fibrils

Amyloid plaque formation is a multistep process that includes several types of amyloid species such as monomers, oligomers, protofibrils, and mature fibrils (Laurén et al., 2009). Aß monomers aggregate to form oligomers, also known as Aß-derived diffusible ligands (ADDLs). Atomic force microscopy (AFM) experiments show that monomers also form flakelike structures that are unstable and disappear quickly dissolving into oligomers or short protofibrils (Lin et al., 2014).

While ADDLs are implicated as the source of AD synaptic disruption preceding the formation of NFT's (Roher et al., 1991; Lambert et al., 1998), multiple *in vitro* studies have shown that ADDLs accumulate around neurons early in AD (Nimmrich & Ebert, 2009; Shankar et al., 2008). The $A\beta_{42}$ species is especially prone to aggregation and is associated with increased neurotoxicity compared to the $A\beta_{40}$ species (Lesné, et al., 2006; Martins et al., 2008; Dubnovitsky et al., 2013). However, the mechanisms underlying oligomeric accumulation are not fully understood due to instability and variations in oligomer aggregation, structure, and toxicity.

Interestingly, different Aß species have varying effects on synapses. This may be attributed to differences in binding affinities (Ferreira, Lourenco, Oliveira & Felice, 2015; Velasco, 2012). Low molecular weight dimers (~8kDa), trimers (~12kDa), and tetramers

(~16kDa) alter synaptic structure and leads to synaptic loss, evidenced by decreased synpatophysin immunoreactivity (Ferreira, Lourenco, Oliveira & Felice, 2015; Reed et al., 2011). In mice injected with high molecular weight Aß oligomers ranging from 50-140kDa, N-methyl-D-aspartate (NMDA) receptors are activated, which leads to neuronal oxidative stress, but not synaptic loss. Furthermore, mice injected with high molecular weight oligomers recovered from memory impairment while mice injected with low molecular weight oligomers did not (Figueiredo et al., 2013). This is supported by evidence demonstrating that low molecular weight Aß oligomers, particularly the trimeric form, induce synapse loss by impairing synaptic plasticity and destabilizing the cytoskeletal elements in *in vivo* studies (Townsend et al., 2006; Shankar et al., 2007; Davis et al., 2011). However, there is no consensus on the impact of Aß species on synapses as several studies show that Aß dimers and trimers do not impact LTP in hippocampal slices (O'Malley et al., 2014) nor do they impact learning and memory when injected into rats intracerebroventricularly (Lesne et al., 2006; Reed et al., 2011).

2.1.12.2 Aß*56-kda

In addition to the $Aß_{42}$ and $Aß_{40}$ species, Aβ*56 has been implicated in AD pathogenesis. Aß*56 consists of four Aβ soluble trimers that accumulate in the extracellular space. Mouse models show that Aß*56 disrupts memory when administered to young rats and has been implicated in impairing memory independently of plaques or neuronal loss (Lesné et al., 2006; Handoko et al., 2013). However, amyloid-β trimers alone were not shown to impair cognition when administered to rats and mice. In human children and adolescent adult neuronal tissue, low levels of Aβ trimers were detected as the only Aβ oligomers. This suggests that trimers are benign at low levels (Lesné et al., 2013). Furthermore, in human cognitively normal adults, Aβ*56 has been seen to increase before amyloid-β dimer or amyloid-β trimer production (Lesné

et al., 2013; Shankar et al., 2008). Therefore, Aβ*56 may contribute to the very early AD pathology.

2.1.13 Apolipoprotein E

2.1.13.1 Background on Genetic Risk

Apolipoprotein E (apoE) is a protein involved in cholesterol transport and is produced primarily by astrocytes and microglia in the central nervous system (Xu et al., 2006; Tai et al., 2014). ApoE is also produced in neurons in small quantities when neurons undergo destabilizing stress-induced conditions (Xu et al., 2006; Mahley, Weisgraber, & Huang, 2009). ApoE is transcribed from the *APOE* gene, which is located on chromosome 19. There are three genotypical variations of apoE: *APOE2, APOE3,* and *APOE4* (Reitz & Mayeux, 2010; Leoni, 2011). Studies have shown that two copies of the *APOE4* allele increase AD risk 15 times. Furthermore, more than half of AD patients have at least one *APOE4* allele (Reitz & Mayeux, 2010; Leoni, 2011, Tai et al., 2014). The *APOE4* allele poses a greater genetic risk for developing late-onset AD compared to *APOE2* and *APOE3*. Additionally, higher levels of insoluble Aß are correlated with *APOE4* compared to *APOE2* and *APOE3* (Corder, Saunders, Pericak-Vance & Roses, 1995; Garai, Verghese, Baban, Holtzman & Frieden, 2014; Tai et al., 2014).

ApoE expression is regulated by ligand activation of nuclear receptors, which can regulate lipid metabolism. These include proliferator activated receptor gamma (PPARγ), liver x receptors (LXRs), and retinoid x receptors (RXRs) (Chawla et al., 2001; Wildsmith, Holley, Savage, Skerrett & Landreth, 2013). LXR and RXR interact and are activated by binding to hydroxylated forms of cholesterol. This process promotes expression of genes that transcribe cholesterol transporters: apoE, ATP-binding cassette transporter 1 (ABCA1), ATP-binding

cassette transporter sub-family G member 1 (ABCG1), and sterol regulatory element binding protein 1c (SREBP1c) (Morales et al., 2008; Wildsmith, Holley, Savage, Skerrett & Landreth, 2013). Furthermore, studies have found when astrocytes are exposed to agonists of LXR, RXR, and PPARγ, there is upregulation of apoE mRNA transcription and apoE synthesis in the astrocyte (Liang et al., 2004; Bu, 2009; Wildsmith, Holley, Savage, Skerrett & Landreth, 2013).

2.1.13.2 ApoE Isoforms

ApoE is a 299–amino acid glycoprotein with a molecular weight of 34.2 kDa that exists in three isoforms in humans: apoE2, apoE3, and apoE4 (Frieden & Garai, 2013). The isoforms differ in structure at amino acids 112 and 158 (Rall et al., 1982; Frieden & Garai, 2013). ApoE2 contains Cys-112 and Cys-158, apoE3 contains Cys-112 and Arg-158, and apoE4 contains Arg-112 and Arg-158 (Brodbeck et al., 2011; Rall et al., 1982). ApoE contains functional domains: N-terminal domain at amino acids 1-191 where the receptor-binding region is located (amino acids 136-150) and the C-terminal domain is located at amino acids 223-299 where the lipid-binding region is located (amino acids 241-272) (Brodbeck et al., 2011; Rall et al., 1982).

ApoE mediates cholesterol processing through isoform-dependent LDLR binding (Tai et al., 2014). Johnson and colleagues (2014) found that in transgenic mice that express apoE2, apoE3, or apoE4, with the absence of human LDLR, there were significant differences in spatial memory, brain cholesterol, and brain apoE levels (Johnson et al., 2014). Furthermore, studies implicate apoE isoform-dependent interactions with Aß that mediate Aß levels directly through the creation of an apoE/Aß complex (Tai et al., 2014; Verghese et al., 2013). Studies in human brain and mouse models have shown that apoE and amyloid-β (Aβ) peptide binding occurs at residues 244–272 of apoE and residues 12–28 of Aβ in an isoform dependent manner (Liu et al.,

2011; Sadowski et al., 2006). Therefore, isoform differences in apoE are implicated in AD pathology and it is imperative to investigate these mechanisms.

2.1.13.2.1 *APOE e2*

The *APOE e2* allele has a prevalence of 7% among the general population (Bennet et al., 2007). *APOE 2/2* genotype, which produces the apoE2 isoform, has a prevalence of 0.7% in the general population (Koopal et al., 2015). Compared to apoE3 and apoE4, the apoE2 isoform has a low affinity for LDLR and is associated with lower risk of sporadic AD susceptibility (Shu et al., 2014). *APOE e2* reduces the overall risk of developing sporadic AD twofold compared to *APOE e3 and APOE e4* (Tai et al., 2015). Furthermore, carriers of the *e2* allele are at increased risk for type III hyperlipoproteinemia (Mizuguchi et al., 2014).

2.1.13.2.2 *APOE e3*

The *APOE e3* allele accounts for approximately 70-80% of the general population. The homozygous *genotype APOE 3/3*, which produces apoE3, is the most common genotype with a prevalence of 50-70% (Frieden & Garai, 2013). The lifetime risk of developing AD with the *APOE 3/3* genotype is approximately 2% at age 75 years and 10% at age 85 years (Zlokovic, 2013; Genin et al., 2011).

Due to its structure, composed of a cysteine residue located on position 112 and an arginine residue on position 158, it is thought that apoE3 provides increased protein stability compared to apoE4 (Zlokovic, 2013). ApoE3 has also been shown to facilitate Aβ clearance through apoE binding to Aß and carrying Aß to LRP1 cell-surface receptors to transport Aß intracellularly through clatherin-mediated endocytosis (Zlokovic, 2013; Sagare et al., 2007). Compared to other isoforms, apoE3 has been shown to be more efficient in Aβ clearance in mice (Kim, Basak & Holtzman, 2009). Furthermore, apoE3 has been shown to promote neuritic

growth and sprouting in cell lines (Nathan et al., 2002; Ruzali, Kehoe & Love, 2012). When neuronal injury occurs, apoE is expressed by neurons and is thought to carry cholesterol for repair and remodeling of injured cell membranes. ApoE3 has been shown to be more efficient at neuronal plasticity and repair compared to other isoforms (Mahley & Huang, 2012).

2.1.13.2.3 *APOE e4*

In contrast to the *ϵ3* allele, the risk for developing AD in *APOE 4/4* individuals approximates 57% at age 70 years and 60% at age 85 years (Genin et al., 2011; Zlokovic, 2013). The *ϵ4* allele is seen in 10-15% of the general population and carriers of this allele account for 65-80% of all AD cases. *APOE 4/4*, which produces apoE4, increases risk for developing sporadic AD significantly by approximately 12-fold compared to *APOE3* (Verghese et al, 2011; Tai et al., 2015).

ApoE4 expression results in delayed Aß clearance compared to apoE2 and apoE3 due to structural differences of functional domains and binding sites (Holtzman, Herz & Bu, 2012; Frieden & Garai, 2013). Structurally, apoE4 possess the unique property of domain interaction between Arg-61, which is located in the amino-terminal domain, and the Glu-255, which is located in the carboxyl-terminal domain. These domain interactions make apoE4 structurally dynamic and prone to proteolytic cleavage where subsequently cleaved apoE4 fragments propagate tau phosphorylation, NFT formation, mitochondrial dysfunction, and neuronal degeneration (Frieden & Garai, 2013; Brodbeck et al., 2011; Mahley and Huang, 2012). Furthermore, apoE4 only displays domain interaction when it is not lipidated or is poorly lipdated (Mahley and Huang, 2012). Lipid depleted apoE is less efficient in clearing Aβ than apoE that are associated with lipids (Hanson et al., 2013).

In terms of synaptic plasticity, learning, and memory, multiple studies show that apoE4 expression attenuates function. Studies in transgenic mice have shown that apoE4 expression increases loss of GABAergic interneurons in the hippocampus, which is detrimental to learning and memory (Bien-Ly et al., 2011). In transgenic mice that expressed apoE4, White and colleagues (2001) found that there was significant impairment in neuronal plasticity and synaptic sprouting by measuring immunoreactivity of synaptophysin and growth association protein 43 (GAP-43) markers after entorhinal cortex lesion compared to apoE3 controls (White, Nicoll, Roses & Horsburgh, 2001; Kim, Yoon, Basak & Kim, 2014). Furthermore, studies demonstrated that transgenic mice with apoE4 expression had significant impairment in working memory during the Morris water maze test and radial arm maze test compared to apoE3 expressing mice (Raber et al., 2000; Hartman et al., 2001).

2.1.13.3 Low Density Lipoprotein Receptor Family

Multiple lines of evidence have shown that the low density lipoprotein receptor family is associated with AD pathogenesis (Holtzman, Herz & Bu, 2012; Johnson et al., 2014; Cheng et al., 2005; Kang et al., 2000; Kanekiyo et al., 2011; Poon and Gariepy et al., 2007). These receptors include low density lipoprotein receptor (LDLR), low density lipoprotein receptor-related protein 1 (LRP1), very low density lipoprotein receptor (VLDL), megalin, and heparan sulfate receptors. In terms of function, the low density lipoprotein receptor family receptors interact with extracellular ligands for the purposes of intracellular internalization of ligands and subsequent degradation by lysosomes (Li, Lu, Marzolo & Bu, 2001). Recent evidence has demonstrated that these receptors also function in cellular signaling through binding of adaptor and scaffolding proteins that contain postsynaptic density protein 95 (PSD-95), discs-large (DLG), or zona

occludens (Z0-1) domains. Signaling functions include the regulation of vesicle trafficking and neurotransmission (Herz, Gotthardt & Willnow, 2000; Li, Lu, Marzolo & Bu, 2001).

In terms of receptor structure, the low density lipoprotein receptor family contains several common structures: 1) ligand-binding repeat segment that contains six cysteine residues and three disulfide bonds, 2) epidermal growth factor precursor repeats that contain six cysteines residues, 3) amino acids with a tetrapeptide, Tyr-Trp-Thr-Asp (YWTD), and 4) NP*X*Y sequence contained in a cytoplasmic tail (Li, Lu, Marzolo & Bu, 2001). Previous evidence demonstrates that the epidermal growth factor precursor repeats and YWTD function in ligand dissociation from the receptor and endosome (Li, Lu, Marzolo & Bu, 2001). Furthermore, each receptor has a transmembrane domain and a cytoplasmic tail, which may play a role in endocytosis signaling (Li, Lu, Marzolo & Bu, 2001; Davis et al., 1987).

These receptors also serve as regulatory components in cholesterol transport and cell membrane remodeling in synaptic plasticity. Dysfunction in synaptic plasticity mechanisms have been implicated in AD and dementia (Gamba et al., 2012; Martin, Dotti & Ledesma, 2010; Johnson et al., 2014). However, these mechanisms remain unclear.

2.1.13.3.1 LDLR

The LDLR family consists of cell surface receptors that recognize ligands functioning in the degradation or recycling pathways (Holtzman, Herz & Bu, 2012). One such ligand is apoE. Because the *APOE e4* allele increases risk for AD, the LDLR family has been widely studied and implicated in the pathogenesis of AD (Kanekiyo & Bu, 2014).

LDLR functions in the regulation of cholesterol in the vasculature and brain. Functional interactions between apoE isoform and LDLR are associated with AD risk. LDLR has a high affinity for apoE and binds to apoE in an isoform-dependent manner where *e4* binds at higher

rates than both *e3* and *e2* (Johnson et al., 2014; Cheng et al., 2005). Studies with plasma and brain apoE levels, neuronal cholesterol, and spatial memory are regulated by LDLR isoform and apoE isoform interactions (Cheng et al., 2005; Retz et al., 2001; Johnson et al., 2014). Furthermore, Johnson and colleagues (2014) found that the lack of LDLR and interaction between apoE4 and LDLR were associated with significant long-term spatial memory dysfunction in transgenic mice expressing apoE4 while lacking LDLR. Therefore, LDLR is critical to cognitive performance and is specific to apoE. Furthermore, LDLR may play a role in AD risk in an isoform dependent manner.

2.1.13.3.2 LRP1

Low-density lipoprotein receptor-related protein 1 (LRP1) is a transmembrane protein expressed in neurons, glial cells, smooth muscle cells, pericyte cells, and parenchyma cells (Bu, 2009). It is implicated in Aß clearance and is a receptor that interacts with apoE, but also has multiple ligands such as Aß, APP, α2-macroglobulin, and RAP (Holtzman, Herz & Bu, 2012; Rebeck et al., 2001). LRP1 serves as a endocytic receptor that endocytoses through interaction with clathrin (Hussain et al., 1999; Rebeck et al., 2001). During endocytosis, LRP1 is internalized into clathrin coated vesicles and subsequently enters endosomal vesicles. Thereafter, LRP1 is undergoes a recycling process where it is transported back to the cell surface (Husain et al., 1999; Li, Lu, Marzolo & Bu, 2001).

In experiments comparing AD versus normal controls, LRP1 expression is increased in younger individuals without AD (Kang et al., 2000). Furthermore, Kang et al. (2000) found that increased LRP1 expression is associated with later onset of pathogenesis in individuals with AD (Kang et al., 2000). There is also evidence that LRP1 is associated with increased APP trafficking through endosomes which increases Aß production (Bu, 2009; Kanekiyo et al., 2011;

Ulery et al., 2000; Cam et al., 2005). LRP1 has also been shown to bind to apoE and co-localize with Aß in AD cortical neurons (Namba et al., 1991; Bu, 2009). Consistent to these findings, a study by Liu et al. (2010) found that deleting the *Lrp1* gene in frontal lobe neurons leads to significant increases in dendritic spine loss, neuronal degeneration, and memory loss in mice (Liu et al., 2010). Additionally, Shibata et al. (2000) also found that applying LRP1 antagonists RAP and LRP1 antibodies against LRP1 caused decreased Aß clearance (Shibata et al., 2000). Therefore, LRP1, like LDLR, plays a critical role in neuronal health and memory.

Structurally, LRP1 differs from other receptors in that the Y*XX*L motif, rather than NP*X*Y, provides a dominant cellular signal for endocytosis (Li, Lu, Marzolo & Bu, 2001). Furthermore, Li and colleagues have demonstrated that endocytosis rates differ significantly among the low density lipoprotein receptor family. LRP1 has been found to have the fastest rate of endocytosis among these receptors and undergoes receptor endocytosis and recycling with or without the presence of a ligand. This may be due to the multiple NP*X*Y and the Y*XX*L signaling structures located on the cytoplasmic tail (Li, Lu, Marzolo & Bu, 2001).

2.1.13.3.3 Heparan Sulfate Proteoglycan

Heparan sulfate proteoglycan (HSPG) is a cell surface receptor found in neurons and other mammalian cell types (Kanekiyo et al., 2011; Poon and Gariepy et al., 2007). Pertaining to AD pathogenesis, HSPG has been seen to co-localize with Aß plaques by binding to the 13–16 region of Aβ (Brunden et al., 1993), which impacts cellular uptake of Aβ in neurons (Brunden et al., 1993; Kanekiyo et al., 2011; van Horssen et al., 2003). It has been suggested that this binding causes a cascade of inflammation and subsequent neurotoxcity (Bergamaschini et al., 2002). Supporting this, Kanekiyo and colleagues (2011) found that overexpression of LRP1 increases Aβ uptake and is mediated by HSPG. Additionally, decreases in HSPG expression are associated

with decreases Aβ cellular uptake (Kanekiyo et al., 2011). Furthermore, heparin, which is an antagonist of HSPG, has been shown to inhibit Aβ uptake into neuronal cells through blocking LRP1 cellular uptake of Aβ (Kanekiyo et al., 2011). Therefore, direct HSPG interaction with Aß may cause clearance neurotoxicity through inflammatory processes, while HSPG interaction with LRP1 may increase Aß uptake and function as a neuroprotective mechanism in AD.

2.1.13.3.4 ApoE Receptor 2 and Very Low Density Lipoprotein Receptor

ApoE receptor 2 (ApoEr2) and very low density lipoprotein receptor (VLDLR) are cell surface receptors located on the postsynaptic terminal (Dumanis et al., 2011). These receptors bind to ligands apoE and Reelin, propagating the Reelin signaling pathway in early neuronal development (Dumanis et al., 2011). The Reelin pathway is involved in learning and memory through facilitation of long-term potentiation (LTP) and increasing dendritic spine density (Dumani et al., 2011; Burrell, Divekar, Weeber & Rebeck, 2014). When Reelin binds to apoEr2 and VLDLR, intracellular Dab1 is phosphorylated by Fyn tyrosine kinase. This leads to phosphorylation of phosphoinositide-3-kinase (PI3 K), which activates protein kinase B (Akt) and leads to inhibition of glycogen synthase kinase 3ß (GSK3ß) (Burrell, Divekar, Weeber & Rebeck, 2014; Holtzman, Herz & Bu, 2012). Experiments in knock-outs of Reelin, apoEr2, and VLDLR results in decreases in GSK3ß phosphorylation and increases in tau phosphorylation, which disrupts microtubule structure and function leading to neuronal dysfunction (Holtzman, Herz, & Bu; Brich et al., 2003; Ohkubo et al., 2003). This evidence suggests that apoEr2 and VLDLR have a role in neuroprotection.

2.1.13.4 Mechanisms of AD Risk

ApoE isoform-dependent differences pose several mechanisms of risk in AD pathology, particularly the apoE4 isoform. The apoE4 isoform is implicated in the both the increase of neurotoxic processes and the loss of neuroprotective function. This is attributed to structural and functional differences between the apoE3 and apoE4 isotypes. Mechanisms pertaining to apoE isoform-dependent AD risk include: 1) increased Aß synthesis and deposition, 2) apoE/Aß complex, 3) impaired clearance of Aß, 4) neuronal injury mediating apoE, 5) mitochondrial dysfunction, and 6) apoE mediated alterations in neuronal cytoskeleton.

2.1.13.4.1 Increased Aß Synthesis and Deposition

ApoE isoform is known to impact Aß deposition and aggregation. ApoE4 is associated with accelerated Aß synthesis and increased amyloid deposition compared to apoE3 and apoE2 (Ye et al., 2005). Ye and colleagues (2005) found that in B103-APP cells with knockdown of LRP1 expression were associated with decreased Aß production compared controls. Additionally, B103-APP cells that were incubated with RAP, which blocks LRP1, showed decreased Aß production (Ye et al., 2005). Furthermore, studies have found that apoE4 domain interaction is associated with increased Aß production. In cultured neuronal cells where ApoE4 domain interaction was disrupted utilizing small molecule compounds that inhibit apoE4 binding to VLDL-like emulsion particles, there was a significant decrease in Aß production (Ye et al, 2005). Additionally, Hori and colleagues (2015) found in APP transgenic mice, early stage Aß protofibrils show a seeding effect that later promotes aggregation of Aß. Furthermore, apoE3 but not apoE4, reduces the deposition induced by promotion of Aß protofibril growth (Hori, Hashimoto, Nomoto, Hyman & Iwatsubo, 2015).

In addition to Aß deposition, apoE isoform-specific effects have been implicated in Aß aggregation. Aß aggregation involves Aß dimer and trimer oligomerization, then oligomer

clustering, and then finally, the fibrilization phase where fibrils form and grow (Garai and Frieden, 2013). Garai and colleagues (2014) monitored Aß aggregation through tetramethylrhodamine (TMR) fluorescence using TMR-labeled Aß and found that apoE binds to Aß oligomers, which leads to Aß fibril growth. Aggregation of Aß1-40 was significantly increased in apoE4 compared to apoE3 and apoE2, implying that the role of apoE may be to bind to oligomers to inhibit conversion to fibrils. Investigators found that apoE4 is not efficient in this process compared to apoE3 and apoE2 showing higher rates of Aß fibril growth and aggregation (Garai, Verghese, Babn, Holtzman & Frieden, 2014). Therefore, the *e4* allele may contribute to increased Aß oligomer production and decreased fibril production.

2.1.13.4.2 ApoE/Aß Complex

In addition to apoE isoform dependent differences in Aβ deposition and aggregation, studies show that apoE binds to Aβ fibrils and oligomers in an isoform dependent manner (Garai, Verghese, Baban, Holtzman & Frieden, 2014). ApoE and Aβ binding is thought to create an ApoE/Aβ complex which has been detected and measured by gel-shift assays of SDS-PAGE, Western blot analysis, density gradient, ultracentrifugation, non-denaturing gradient gel electrophoresis, co-immunoprecipitation, size exclusion chromatography and gel filtration, and solid phase binding assays (Tai et al., 2014). ApoE and Aβ binding is mediated by apoE lipidation. Studies show that when apoE is lipidated, apoE3 complexes Aβ with at higher rates than apoE4 (LaDu et al., 1995; Tai et al., 2014; Garai, Verghese, Baban, Holtzman & Frieden, 2014). Furthermore, apoE4 is thought to be less lipidated than apoE3 due to conformational instability of apoE4 (Tai et al., 201& Narayanaswami, 2008). *In vitro* studies demonstrate that degradation of apoE4 by glia is associated with reduced cholesterol release compared to apoE3 (Riddell et al., 2008; Tai et al., 2014). Additionally, Aβ clearance rate has been shown to be

slower in apoE4 compared to apoE3 (Tai et al., 2014; Castellano et al., 2011). Therefore, apoE4 is not as efficient in clearing Aβ compared to apoE3 possibly due to poor lipidation status and structural instability that leads to lower apoE4/Aβ complex (Tai et al. 2014).

Although studies have shown evidence of an apoE/Aβ complex, studies often add Aβ concentrations that exceed physiological values in humans, resulting in increased apoE/Aβ complex *in vitro* (Tai et al., 2014). Verghese and colleagues (2013) found that utilizing physiologically relevant ratios of Aβ:apoE in human CSF and plasma brain homogenates from humans and FAD-Tg mice produced minimal amounts of apoE/Aβ complex (Verghese et al. 2013). This data suggests that apoE and Aβ do not directly interact in the extracellular environment, but rather through LRP1 mediating the interaction. Investigators suggest that apoE and Aβ compete for LRP1 binding sites, which may account for the perceived apoE regulation of Aβ levels (Verghese et al., 2013). Therefore, the existence of apoE/Aβ complex *in vivo* is controversial. More studies with *in vivo* models with physiologically accurate concentrations of Aβ and apoE need to be conducted.

2.1.13.4.3 Clearance of Aß

Sporadic late onset AD is related to reduced Aß clearance in the synapse. Because Aß aggregation is thought to contribute to AD pathology, investigating mechanisms of dysfunctional Aß clearance is imperative in finding therapeutic targets. Potential Aß clearance processes include glial clearance, pathways toward autophagy, enzymatic dissolution of Aß, and blood brain barrier clearance.

2.1.13.4.3.1 Glial Clearance

Astrocytes and microglia are involved in Aß clearance via phagocytosis, which is thought to be mediated by apoE (Wildsmith, Holley, Savage, Skerrett & Landreth, 2013). Astrocytes are the most numerous cells in the human brain and function in regulating the blood brain barrier, synaptogenesis, and calcium concentration. Astrocytes have been shown to surround Aß plaques and are thought to be a neuroprotective mechanism. In response to interleukin 1 (IL-1) release, astrocytes increase expression of proteins that bind to Aß such as α1-antichymotrypsin (α1-ACT), apoE, and C3 (Akiyama et al., 2000). Furthermore, multiple studies have shown that astrocytes in adult mice phagocytose extracellular Aß (Mandrekar et al., 2009; Koistinaho et al., 2004). Although astrocyte clearance of Aß via phagocytosis is not as efficient as in microglial cells, this pathway may contribute to Aβ clearance.

Microglia, on the other hand, are well recognized as the first response to Aß accumulation. The main functions of microglia include repairing damage to the neuropil and rapidly responding to pathogens by releasing cytokines (Theriault, ElAli, and Rivest, 2015). Moreover, microglia have been shown to surround Aß plaque and are thought to be directly involved with Aß clearance through phagocytosis (Malm et al., 2005). It has been shown that microglia respond to Aß via receptor recognition utilizing toll like receptors (TRL), receptors for advanced end glycation products (RAGE), and LRP1 (Theriault, ElAli, and Rivest, 2015). Terwel and colleagues (2011) treated primary astrocytes from APP 23 mice with a LXR agonist, which regulates apoE expression, and saw increased microglial phagocytosis of Aß plaques. Investigators also saw associated increases in ABCA1, apoE levels in astrocytes, and apoE lipidation (Terwel et al., 2011). Furthermore, Lee et al. (2012) found that apoE modulates microglial cholesterol levels, which promotes Aß trafficking into microglial lysosomes for

degradation (Lee et al., 2012). Thus, evidence suggests that glial cell activation is mediated by apoE and involved in Aß intracellular degradation.

2.1.13.4.3.2 Autophagy

Autophagy is a process where cytoplasmic materials such as protein aggregates and dysfunctional organelles are degraded by lysosomes (Salminen et al., 2013). There are three types of autophagy pathways: macroautophagy, microautophagy, and chaperone-mediated autophagy (Salminen et al., 2013; Todde et al., 2009). Microautophagy and chaperone-mediated autophagy involve the direct transfer of cytoplasmic materials into the lysosome, while macroautophagy requires isolation of cytoplasmic materials into autophagosomes (Salminen et al., 2013).

Macroautophagy has been the most widely investigated type of autophagy and is shown to be impaired in AD due to beclin1/Vps34 complex dysfunction, which is an important element of autophagosome formation (Ronan et al., 2014). The beclin1/Vps34 is composed of beclin 1 adaptor protein complex that controls onset and degree of autophagy, and Vps34 that is a class III isoform of phosphoinositide 3-kinase (PI3K) (Ronan et al., 2014; Salminen et al., 2013). Recent studies show that increased expression of Beclin 1 facilitates APP breakdown that subsequently decreases Aß deposition in AD mouse models (Jaeger et al., 2010). Also, reductions or impairment of beclin 1 are associated with dysfunctional APP processing, increased Aß aggregation, and impaired autophagy (Salminen et al, 2013). The disruption of beclin 1 mediated autophagy leads to the congestion of intracellular Aß aggregates and eventually, neuronal death (Guglielmotto et al., 2014). In non-pathological states, it is thought that apoE carries Aß to LRP1 cell surface receptors, which induces Aß autophagy and degradation that is mediated by clathrin (Pflanzer, Kuhlmann, & Pietrzik, 2010).

2.1.13.4.3.3 Enzymatic Clearance

Another mechanism of Aß clearance is degradation of neurotoxic Aß oligomers with Aß degrading enzymes such as insulin degrading enzyme, endothelin-converting enzyme-1 (ECE-1), ECE-2, and neprilysin (NEP) (Bohm et al., 2015; Qiu et al., 1998; Miners et al., 2008). NEP, ECE-1 and ECE-2 are type II membrane bound zinc metalloproteases and are found in areas of the brain that are relevant to late AD (Pacheco-Quinto, Herdt, Eckman & Eckman, 2013). These Aβ-degrading enzymes are found in multiple compartments such as lysosomes, endosomes, late endosomes, extracellular space, intracellular space, and the plasma membrane (Eckman and Eckman, 2005). In a study using the hippocampus of aged 3xTg-AD mice at 12 and 18 months, Oddo and colleagues (2006) stained intraneuronal Aß and measured NEP levels and found that NEP decreased with age (Oddo, Caccamo, Smith, Green & LaFerla, 2006). Moreover, Pacheco-Quinto and colleagues (2013) found that Aβ concentration is significantly increased in ECE-1 and ECE-2 knockout mice.

2.1.13.4.3.4 Blood Brain Barrier Clearance

The blood brain barrier (BBB) functions to maintain brain homeostasis and has selective permeability to water and lipid-soluble molecules through passive transport. Structurally, the BBB is composed of endothelial cells that are connected to one another with tight junctions. The BBB is also surrounded by pericytes, astrocytes, and microglia that are involved in BBB clearance of Aß (Wildsmith, HolleySavage, Skerrett & Landreth, 2013). Mechanisms of BBB Aß clearance have been widely investigated, particularly those involving phosphatidylinositol-binding clathrin assembly (PICALM) protein and ATP-binding cassette (ABC) transporter clearance.

2.1.13.4.3.4.1 PICALM

Aβ clearance across the BBB through active transport is conducted by specific cell surface receptors. Phosphatidylinositol-binding clathrin assembly (PICALM) protein is expressed in neurons and brain capillary endothelial cells in the BBB (Zhao et al., 2015). It is implicated in mediating Aβ clearance across the BBB and internalization of cell surface receptors (Zhao et al., 2015). Additionally, PICALM is thought to reverse damaging mechanisms of Aβ on clathrin-mediated endocytosis through APP trafficking towards autophagosomes. Zhao and colleagues (2015) suggest that PICALM brings Aβ towards a transcytotic pathway where PICALM/clathrin-dependent endocytosis of Aβ-LRP1 complex by endothelial cells. This process is thought to reduce Aβ

production (Tian, Chang, Fan, Flajolet & Greengard, 2013; Zhao et al., 2015). Through co-immunoprecipitation analysis after Aβ40 treatment in CSF Zhao and colleagues (2015) found that endocytosis of Aβ/LRP1 complex requires both PICALM and clathrin. Additionally, investigators found that *Picalm* deficient mice had diminished Aß40 and Aß42 transfer across the BBB compared to normal controls (Zhao et al., 2015).

2.1.13.4.3.4.2 ABC Transporters

The ATP-binding cassette (ABC) transporter family actively transports lipophilic and amphipathic substances across cell membranes using ATP hydrolysis (ElAli & Rivest, 2013; Abuznait & Kaddoumi, 2012). In the CNS, ABC transporters are expressed by endothelial cells, astrocytes, microglia, and neurons (Abuznait & Kaddoumi, 2012). It is thought that ABC transporters function to protect the brain from exogenous molecules and maintain the BBB. In

particular, ABC transporter sub-family B member 1 (ABCB1) and ABC transporter sub-family A member 1 (ABCA1) are involved in Aß processing and are implicated in AD pathogenesis (ElAli & Rivest, 2013; Abuznait & Kaddoumi, 2012).

ABCA1 has high expression in astrocytes, microglia, neurons, and capillary endothelial cells of the brain. It regulates cholesterol efflux from the brain and mediates apoE lipidation, which impacts apoE-Aß binding (ElAli & Rivest, 2013). ABCA1 does not directly interact with Aß, but rather, interacts directly with apoE by loading lipids onto apoE (lipidation) (Nordestgaard, Tybjærg-Hansen, Nordestgaard & Frikke-Schmidt, 2015). Previous studies have shown decreasing levels of ABCA1 or deletion of ABCA1 is associated with decreased levels of apoE that is poorly lipidated (Wahrle et al., 2004; Hirsche-Reinshagen et al., 2004). Additionally, several *in vitro* studies show that a decrease in ABCA1 is associated with increased Aß plaque deposition and reduced Aß clearance (Fukumoto et al., 2002; Kuhnke et al., 2007). In postmortem human studies, ABCA1 was significantly increased in the hippocampus of AD brains versus normal controls (Akram, Schmeidler, Katsel, Hof, & Haroutunian, 2010). However, in contrast, Kim and colleagues (2007) show that a decrease in ABCA1 inhibits APP processing and reduces Aß production. Therefore, ABCA1 is implicated in AD pathogenesis and there is a need for further investigation on its impact on Aß production.

Unlike ABCA1, ABCB1 directly binds to Aß and is predominantly expressed on the luminal side of brain endothelial cells (ElAli & Rivest, 2013). In human brain, ABCB1 decreases in aging and also decreases expression in AD compared to normal controls (Silverberg et al., 2010; Vogelgesang et al. 2002; ElAli & Rivest, 2013). Furthermore, *in vitro* studies have shown that ABCB1 inhibition decreases Aß clearance, leading to elevated levels of Aß aggregation

(Lam et al., 2001; Kuhnke et al., 2007). This evidence suggests that ABCB1 expression in brain endothelial cells impacts AD pathogenesis through Aß transport and clearance.

2.1.13.4.4 Neuronal Injury and ApoE

ApoE plays a pertinent role in the repair of injured neurons and neuronal remodeling (Lahiri, 2004). Because of these functions, investigations aim to elucidate mechanisms in neuronal injury induction of apoE and associations of isoform-dependent AD risk. Several *in vitro* studies show that neuronal injury induces glial production and release of apoE (Petegnief, Saura, de Gregorio-Rocasolano & Paul, 2001; Aoki et al., 2003). Petegnief and colleagues (2001) treated neuron and glial co-cultures with NMDA to induce neuronal injury. This caused initial astrogliosis and microgliosis, followed by an increase apoE messenger RNA expression and an increase in intracellular apoE (Petegnief, Saura, de Gregorio-Rocasolano & Paul, 2001).

Because apoE has been found to co-localize with Aß plaque and microglia in AD, studies suggest that apoE may also regulate innate immunity in response to neuronal injury (Uchihara et al., 1995). Innate immunity defends the body through non-pathogen-specific pathways that utilize the complement cascade and a toll-like receptors (TLRs). Aβ has been shown to directly bind to the C1q protein, which activates the complement cascade (Keene, Cudaback, Li, Montine & Montine, 2011). Furthermore, studies in AD mouse models show that C3 and C5 proteins of the complement system contribute to altered plaque deposition are implicated in altered plaque deposition (Fonseca et al., 2009).

However, the innate immune response may lead to production of neurotoxic species. Maezawa and colleagues (2006) found that neurotoxicity occurs from the innate immune response via microglial activation of p38 MAPK and is increased with the apoE4 isoform compared to apoE3 and apoE2 microglia (Maezawa et al., 2006). Investigators also found that

cell cultures of microglia expressing apoE4 secrete more neurotoxic species such as nitric oxide (NO), TNFα, and IL-6 compared to apoE3 and apoE2 expressing microglia (Keene, Cudaback, Li, Montine & Montine, 2011). This evidence indicates that apoE isoform play a role in neuronal injury by mediating microglial-induced neurotoxicity and is produced in response to neuron injury.

2.1.13.4.5 ApoE Fragment and Mitochondrial Dysfunction

The C-terminal truncated apoE(1-272) fragment has been found to be associated with mitochondrial dysfunction in cultured neuronal cells and transgenic mice (Huang et al., 2001; Harris et al., 2003; Brecht et al., 2004; Dafnis et al., 2011). Additionally, the apoE4 isoform is more prone to proteolytic cleavage and fragmentation compared to apoE3 in neurons (Wellnitz et al., 2005). Cleavage sites are thought to be located at residues 268/272, 187, or after residue 160 (Harris et al., 2003; Wellnitz et al., 2005, Cho et al., 2001, Dafnis et al., 2011). Furthermore, apoE (1-272) fragment accumulation increases in an isoform-dependent and age-dependent manner.

Studies have found that the fragment is significantly increased in older transgenic mice expressing apoE4 compared to apoE3 mice and is associated with memory deficits (Brecht et al., 2004; Harris et al., 2003). In addition, apoE4 (1-272) fragments aggregate with tau in mitochondria, which causes mitochondrial dysfunction (Huang et al., 2001; Chang et al., 2005; Nakamura et al., 2009). Nakamura and colleagues (2009) found that the apoE4 (1-272) fragment significantly reduces mitochondrial enzyme function by binding to complex UQCRC2 cytochrome C1 in mitochondrial complex III and COX IV 1 in mitochondrial complex IV

compared to non-fragmented apoE4 in neuronal cell cultures (Nakamura et al., 2009). Furthermore, Caspersen and colleagues (2005), mitochondrial complex III and IV activity is reduced in AD human and mouse brain compared to normal controls (Caspersen et al., 2005). Mitochondrial complexes III and IV function in ATP synthesis and maintaining membrane potential of mitochondria, which are important to cell survival (Nakamura et al., 2009). Therefore, dysfunction of these processes induced by apoE4 (1-272) fragment are thought to contribute to neuronal toxicity in AD.

2.1.13.4.6 ApoE-Mediated Cytoskeleton Changes

In addition to disruption of mitochondrial processes, apoE4 is associated with cytoskeletal changes. The cytoskeleton is comprised of microtubules, actin filaments, and intermediate filaments and functions to maintain cellular structure, facilitate cell division, and aids in intercellular transport (Bamburg & Bloom, 2009). Tau protein, which is a type of microtubule associated protein (MAP), is a relevant cytoskeletal element in AD pathogenesis and is altered by apoE. *In vitro* experiments involving AD neuronal cell cultures with additive C-terminal truncated apoE4 (1-272) fragments show cytosolic formation of phosphorylated tau and phosphorylated neurofilaments (1-272) (Huang et al., 2001; Ljungberg et al., 2002). Furthermore, in human neuronal cell cultures, apoE4 overexpression results in increased phosphorylation of tau and may be mediated by increased GSK3ß activation (Tesseur et al., 2000). Tesseur and colleagues (2000) also found that apoE4 interferes with microtubule formation and decreases β tubulin in neuronal cell cultures. This evidence suggests that apoE isoform, particularly apoE4, contributes to cytoskeletal dysfunction and instability in AD.

2.1.13.5 Apolipoprotein Dependent Lipidation

ApoE is the main cholesterol transporter in the brain and undergoes endocytosis by LDL receptors to deliver cholesterol to cells. Once apoE is endocytosed, it is either degraded or transported to the cell surface (Rensent et al., 2000; Kim, Basak & Holtzman, 2009). During this process, cholesterol is released from apoE and is utilized by the cell for various cell maintenance functions such as synaptogenesis and membrane repair (Kim, Basak & Holtzman, 2009).

Studies in animal models and neuronal cell culture have shown that apoE functions in clearing Aβ and this is related to its ability to bind to lipids (Fan et al., 2009; Tokuda et al., 2000; Hanson et al., 2013). ApoE that is poorly lipidated or not lipidated is less efficient in clearing Aβ than apoE that is associated with lipids (Hanson et al., 2013). In the human brain, Aβ peptide can be bound to apoE lipid carriers, directly bound to lipids, or unbound. Unbound Aβ is more likely to form neurotoxic oligomers and therefore, examining Aβ and apoE in relation to lipid homeostasis is imperative in understanding AD pathology (Hanson et al., 2013).

Studies in human and mouse models show that lipidation is dependent on apoE isoform (Hu et al., 2015; Hanson et al., 2013). Hu and colleagues (2015) utilized apoE-targeted replacement (TR) mice that were transfected with adeno-associated viral serotype 8 (AAV8) viral vectors expressing *APOE e2* and *e4* alleles. In apoE4-TR mice expressing apoE4, there were increased apoE4 levels, decreased apoE lipidation, decreased apoE-associated cholesterol, and increased Aβ accumulation compared to mice that expressed apoE3 and apoE2. Moreover, in mice overexpressing apoE2, apoE lipidation was increased while Aβ accumulation was decreased (Hu et al., 2015). Therefore, the apoE4 expression has deleterious effects in AD pathology through reduced lipidation, which increases Aβ accumulation. Furthermore, based on

the study conducted Hu and colleagues (2015), altering apoE isoform expression by inducing overexpression apoE2 levels may serve as a therapeutic target in AD to reduce Aß accumulation and increase lipidation.

2.1.13.6 Apolipoprotein Effects on Tau

In addition to apoE mediation of Aß accumulation, there is mounting evidence of apoE association with tau in AD (Andrews-Zwilling et al., 2010; Liraz, Boehm-Cagan & Michaelson, 2013). Tau is a microtubule-associated protein (MAP) that is hyperphosphorylated in AD (Huang et al., 2010; Lewis et al., 2000). Tau hyperphosphorylation causes assembly of helical filaments that form NFTs, which are a pathological hallmark in AD and associated with neuronal loss (Hyman et al., 2013; Huang et al., 2010). ApoE has been found in NFTs in AD brains, suggesting apoE and tau interaction in AD (Huang, 2010).

There are apoE isoform differences in apoE associated tau interactions in AD. In *in vitro* studies, the N-terminal domain of apoE3 has been shown to bind to non-phosphorylated tau, forming a stable complex with tau by irreversibly binding to the microtubule-binding repeat regions of tau (Strittmatter et al., 1994; Huang et al. 2010). ApoE4, on the other hand, is associated with inducing tau phosphorylation in neurons, which is a hallmark of AD pathology (Huang et al., 2010). Recent studies demonstrate that C-terminal truncated apoE4 fragments lead to the formation of NFT-like inclusions in neurons, suggesting that apoE4 fragments contribute to cytoskeletal dysfunction in AD (Huang et al., 2010; Ljungberg et al., 2002).

Andrews-Zwilling and colleagues (2010) found that GABAergic interneuron loss and memory deficits were significantly increased compared to controls in transgenic mice with C-terminal truncated apoE4 fragment, which was mediated by age and tau. Because inhibitory synaptic plasticity occurs during learning and memory tasks, with the rapid release of GABA

from cortical neurons and sustained GABA release from hippocampal interneurons (Perrin et al., 2009), loss of these processes coupled with cognitive deficits in learning and memory tasks suggests AD pathology. Additionally, Andrews-Zwilling and colleagues (2010) found significant tauopathy increases occurred earlier in apoE4 fragment mice compared to controls, which correlated with deficits in memory tasks (Andrews-Zwilling et al., 2010).

Liraz, Boehm-Cagan and Michaelson (2013) investigated the early effects of apoE isoform on tau phosphorylation in apoE4-expressing mice (Liraz, Boehm-Cagan & Michaelson, 2013). Investigators found that at 1 month of age, tau hyperphosphorylation is greater in apoE3 mice compared to apoE4 mice. At 4 months of age, tau hyperphosphorylation is significantly lower in apoE3 mice compared to apoE4 mice. From these experiments, Liraz and colleagues (2013) concluded that apoE isoform contributes to a biphasic effect in tau phosphorylation, which begins early in disease progression (Liraz, Boehm-Cagan & Michaelson, 2013). However, more evidence is needed to elucidate the specific molecular mechanism underlying apoE4 induced tau phosphorylation, GABAergic interneuron loss, and biphasic pattern of tau phosphorylation in AD.

2.1.13.7 Apolipoprotein E Related Therapeutics

Due to the confirmatory evidence that apoE plays a significant role in AD pathogenesis, studies seek to alter apoE expression, structure, and function as possible therapeutic targets for AD. To date, there have been several therapeutic agents identified that target apoE in AD. However, none of these has been confirmed as a definitive treatment for AD due to issues with safety profile and efficacy in humans.

2.1.13.7.1 Bexarotene

Bexarotene (Targretin) is a highly selective retinoid X receptor agonist that is able to cross the BBB to increase apoE expression in astrocytes and microglia (Balducci et al., 2015; Cramer et al., 2012). Bexarotene was approved by the U.S. Food and Drug Administration (FDA) in 1999 for the treatment of cutaneous T-cell lymphoma and has since been used off-label for the treatment of non-small cell lung cancer and breast cancer (Cramer et al., 2012; Dragnev et al., 2007; Esteva et al., 2003). In 2012, Cramer and colleagues reported that Baxarotene facilitated Aß clearance in AD mice. Investigators treated primary microglia and astrocytes with Bexarotene, which showed increases in expression of apoE, ABCA1, and ABCG1. This led to significant Aß42 clearance within 6 hours of administration and an overall 25% reduction of Aß40 and Aß42 levels within 70 hours of administration. A return to baseline of Aß40 and Aß42 levels occurred after 84 hours after Bexarotene treatment (Cramer et al., 2012). Although these results are promising, different labs were unsuccessful in attempting to replicate the results (Tesseur et al., 2013; Veeraraghuvalu et al., 2013; Price et al., 2013; Bohm et al., 2015).

2.1.13.7.2 miRNA-33 Antisense Oligonucelotide Treatment

A microRNA (miRNA) is a non-coding RNA molecule that is approximately 15-22 nucleotides in size. MiRNAs regulate gene expression after transcription by binding to mRNAs to inhibit translational functions or facilitate mRNA degradation (Bartel, 2009; Jan et al., 2015). Because of these functions, miRNAs have been suspected in contributing to AD pathogenesis. In particular, miR-33, has been investigated due to its association with downregulation of ABCA1 expression. It does this through interaction with the 3' region of the *ABCA1* gene, which impacts apoE lipidation (Jan et al., 2015).

Studies in mice and primates show that miR-33 antisense oligonucleotide (ASO) inhibits miR-33, which increases expression of ABCA1 (Jan et al., 2015; Rayner et al., 2011). The increased expression of ABCA1 facilitates increased apoE lipidation and subsequent Aß clearance in AD (Wahrle et al., 2008). Furthermore, Jan and colleagues (2015) utilized *in vitro* astrocyte cultures to demonstrate that inhibiting miR-33 with miR-33 ASO increases apoE and cholesterol secretion. Investigators also showed that infusion of miR-33 ASO into intraventricular regions of mouse brain increased ABCA1 expression (Jan et al., 2015). Based on this evidence, miR-33 ASO treatment demonstrates promising approach towards treating AD. Further *in vivo* studies are needed to investigate the potential applications of miR-33 ASO in human AD.

2.1.13.7.3 Small-Molecule Structure Correctors Targeting apoE

In addition to altering apoE expression, recent studies seek to change apoE utilizing small-molecule structure correctors. Small-molecule structure correctors are able to interchange single amino acids and alter dysfunctional protein structures that contribute to cellular toxicity (Mahley and Huang, 2012). This is a promising therapy for disorders involving misfolded proteins such as cystic fibrosis, lysosomal storage disease, p53 mediated cancers, and neurological disorders such as AD (Kalid et al., 2010; Mahley and Huang, 2012).

Variations between apoE4 and apoE3 structure account for differences in AD risk (Holtzman, Herz & Bu, 2012; Frieden & Garai, 2013; Mahley and Huang, 2012). In apoE4, Arg-61 is located in the amino-terminal domain and Glu-255 is located in the carboxyl-terminal domain. These are thought to ionically interact with one another (Mahley and Huang, 2012). These domain interactions mediate apoE4 preferential binding to VLDL and also create a highly dynamic structure in apoE4 (Holtzman, Herz & Bu, 2012). ApoE3 also undergoes ionic

interaction to some degree and possess a dynamic structure, but to a lesser extent compared to apoE4 (Mahley and Huang, 2012). Therefore, recent studies aim to stabilize apoE4 structure through implementation of small-molecule structure correctors that mimic apoE3 structure.

Through X-ray crystallographic structure analysis of apoE4 and DOCK screening, Ye and colleagues (2005) identified eight small molecules that disrupt apoE4 domain interaction without impacting apoE3 binding. Investigators found that the GIND-25 and GIND-105 compounds significantly reduced apoE4 domain interaction and decreased Aβ comparable to levels seen in apoE3 controls (Ye et al., 2005). Mahley and Huang (2012) investigated apoE4 conversion into a molecule that is structurally similar to apoE3 using compounds that bind and inhibit regions where domain interaction occurs. Investigators observed that apoE4 is misfolded due to domain interaction and is partially cleaved by proteases because it is recognized as an abnormal protein (Mahley and Huang, 2012). Partial proteolytic cleavage of apoE4 creates the neurotoxic apoE4 fragment. To inhibit this processes Mahley and Huang (2012) applied a small-molecule structure corrector to neuronal cells that express apoE4, which led to significantly decreased proteolytic cleavage and reduced apoE4 fragments (Mahley and Huang, 2012). This evidence suggests that small-molecule structure correctors may be a possible therapeutic agent targeting apoE4 in AD by structurally stabilizing it. However, more studies involving human samples must be conducted to demonstrate safety profile and efficacy of therapy.

2.1.14 Cholesterol Metabolism in Alzheimer's disease

2.1.14.1 Cholesterol Synthesis and Processing in the Brain

As mentioned previously, apoE is the primary transporter of cholesterol in the brain. Both apoE and HDL cholesterol are produced by astrocytes and subsequently bind to each other through ABCA lipidation. This apoE/HDL cholesterol complex is hypothesized to bind to Aß to

clear it from the synapse (Arold et al., 2012; Gylys et al., 2007). Furthermore, apoE that is not bound to HDL cholesterol has poor Aß binding and reduces Aß clearance (Murphy et al., 2013). Therefore, it is important to explore cholesterol dynamics in the brain due to its role in apoE lipidation and possible Aß clearance.

Cholesterol synthesis in the brain occurs *de novo* and is independent of cholesterol synthesis that occurs systemically (Djelti et al., 2015; Vance, 2012). Cholesterol in the brain exists in two forms: the unesterified form, which accounts for 95% of cholesterol in the brain, and the esterified form, which constitutes 5% of total cholesterol. Unesterified cholesterol, also referred to as free cholesterol, is able partition into the cell membrane of neurons because it contains 3-ß-OH group (Dietschy, 2009). Cholesterol in its free form is thought to function in cellular membrane fluidity, electrical transduction, structure, repair, and permeability (Dietschy, 2009).

Free cholesterol can be converted to esterified cholesterol by acyl-coA:cholesterol acyltransferase (ACAT1:ACAT2) enzymes in human brain (Murphy et al., 2013). Unlike free cholesterol, esterified cholesterol does not contain a 3-ß-OH group and is not found in the cell membrane. Instead, esterified cholesterol contains a hydrophobic core and forms intracellular lipid droplets. Lipid droplets are thought to function in lipid storage and cholesterol homeostasis intracellularly (Murphy et al., 2013; Bryleva et al., 2010). Esterified cholesterol is converted to 24S-hydroxycholesterol (24S-OH-C) by cholesterol 24-hydroxylase enzyme (CYP46A1). 24-OH-C is able to cross the blood brain barrier (BBB) and exit to be processed and excreted by the liver (Bryleva et al., 2010). Additionally, esterified cholesterol can be converted back to free cholesterol through cholesterol hydrolase (Petrov, Kasimov & Zefirov, 2016).

2.1.14.2 Cholesterol in AD

A growing body of literature demonstrates an association between dysfunctional cholesterol homeostasis and AD (Djelt et al., 2015). Although free cholesterol, which constitutes 95% of the species in the brain, is thought to function in membrane repair and synaptic plasticity, Schneider and colleagues (2006) have found that high levels of free cholesterol can contribute to inefficient Aß clearance due to instability of the membrane (Schneider, Schulz-Schaeffer, Hartmann, Schulz & Simons, 2006). Furthermore, increased levels of free cholesterol is thought to obstruct trafficking of proteins through the membrane which may lead to reduced Aß clearance and synaptic function in AD (Hicks, Nalivaeva & Turner, 2012).

Several studies have sought to manipulate cholesterol dynamics in the brain to investigate potential cholesterol mechanisms in AD. In a study conducted by Hutter-Paier and colleagues (2004), APP transgenic mice containing the London and Swedish mutations were treated non-specific ACAT inhibitors. This reduced the accumulation of Aß plaques by 88%-99%, membrane-bound insoluble Aß levels by 83%-96%, and esterified cholesterol by 86% compared to untreated mice. Investigators also found that spatial learning slightly improved in mice treated with ACAT inhibitors (Hutter-Paier et al., 2004).

Bryleva and colleagues (2010) ablated ACAT1 in triple transgenic mice and saw a 60% reduction in human APP and proteolytic fragments. Moreover, investigators observed improved cognitive function in mice with cognitive deficits. Additionally, ACAT1 ablation caused an increase in 24-S-OH and cholesterol in neurons (Bryleva et al., 2010). This evidence strongly supports that cholesterol dynamics play a key role in AD and suggests that increases esterified cholesterol and free cholesterol may contribute to neurotoxicity in AD.

2.1.14.2.1 Lipid Rafts

Lipid rafts are microdomains of cell membranes that consist of a high concentrations of free cholesterol and tightly packed sphingolipid acyl chains (Pike, 2006; Simons & Gerl, 2010). They are biochemically characterized as insoluble in non-ionic detergents at low temperatures and are also known as detergent-resistant membranes (Rushworth & Hooper, 2011; Simmons & Gerl, 2010). Lipid raft domains enable proteins and lipids to partition the membrane and facilitate cell-signaling functions (Rushworth & Hooper, 2011).

Pertaining to AD pathogenesis, lipid rafts have been found to increase the production of Aß peptide and Aß aggregation, forming neurotoxic Aß oligomers (Rushworth & Hooper, 2011). This is related to lipid raft interaction with ß-secretase and increasing APP production (Gylys et al., 2007; Rushworth and Hooper, 2011). Studies have demonstrated that Aß oligomers aggregate and binding to lipid raft proteins such as cellular prion protein and glutamate receptors promoting synaptic loss (Rönicke et al., 2011; Alberdi et al., 2010). Furthermore, free cholesterol depletion in the membrane is associated with reduced Aß aggregation in hippocampal neurons (Schulz-Schaeffer, Hartmann, Schulz & Simons, 2006). This evidence points to the detrimental impact of increased lipid raft and membrane cholesterol concentrations leading to synaptic loss in AD.

Although the studies mentioned previously imply lipid raft involvement in AD pathogenesis, recent studies suggest that lipid rafts serve protective role against neurodegeneration. Hering and colleagues (2003) found that lipid rafts in cultured neurons are associated with AMPA receptors and that the depletion of lipid rafts in the membrane leads to instability of AMPA receptors and loss of inhibitory and excitatory synapses. Furthermore, investigators have found that decreases in lipid rafts are associated with enlarged, structurally

malformed, and dysfunctional synapses suggesting that lipid rafts function in the maintenance of synapses (Hering, Lin & Sheng, 2003).

Reinforcing these findings, a study conducted by Cecchi and colleagues (2009) on human SH-SY5Y neuroblastoma cells found that the content of membrane cholesterol and neurotoxic membrane ADDLs are inversely related. Investigators utilized confocal microscopy and found that Aß ADDLs produce large cavities in lipid rafts that were depleted of cholesterol while cholesterol-enriched lipid rafts had reduced size and depth of cavities (Cecchi et al., 2009). Cecchi and colleagues (2009) concluded that membrane cholesterol contained in lipid rafts serves as a protective mechanism by decreasing pores forming in the membrane. Due to contradicting findings and conclusions regarding lipid rafts and free cholesterol neurotoxic effects in AD, it is imperative to investigate cholesterol dynamics and their relationship to synaptic loss in AD.

Chapter 3

Framework

3.1 Underlying Philosophy

A structured theoretical framework will be used to explore synaptic changes in AD versus normal controls. The underlying philosophical approach to this study is empiricism. Historically, empiricism developed during the 17th century Enlightenment when traditional beliefs were challenged and intellectual debates on the origin of knowledge ensued (Rodgers, 2005). According to Monti and Tingen (1999), empiricism enables hypothesis testing, comparison of interventions, generalization, and development of confidence intervals. Additionally, empiricists assert that the origin of human knowledge is derived from sensory experience and upholds two major tenets of deductive reasoning and substantiation of theoretical claims (Weiss, 1995; Rodgers, 2005). By upholding the major tenets of deductive reasoning and substantiation of theoretical claims, empiricism aims to measure, predict, and explain phenomenon (Rodgers, 2005).

Empiricism offers significant contributions to the development of nursing knowledge and is consistent with nursing research in several aspects. Because nurses are involved in evaluation of health status through visual and physiological data collection, utilizing empirical methods for gathering sensory data is invaluable to nursing research (Rodgers, 2005). According to Weiss (1995), empiricism relies on three assumptions: predictability versus universality, knowledge to improve nursing practice, and analysis and synthesis. In empiricism, reasonable predictions are possible and can provide nurses with explanations of human responses associated with health (Weiss, 1995). Overall, empiricism provides a systematic approach of clarification and aids in the development of predictions that are consistent with nursing knowledge development.

Moreover, empiricism is useful in substantiating claims in health care and generating new nursing theory (Weiss, 1995).

Empirical philosophy and approaches support the aims of this study. The specific aims of this study are to identify variables that differentiate AD brain from normal brain. Variables of this study serve as empirical indicators that can be quantified. This data will be used to deduce rational predictions regarding AD progression. Through applying empirical approaches of deductive reasoning, findings from this study will build on knowledge of AD pathogenesis, which is pertinent to nursing.

3.2 Definitions of Study Concepts and Variables

Defining study concepts and variables is pertinent to conducting research nursing to provide consistency within this study. Additionally, it is necessary to define and describe measurement of variables to compare the results of this study to other similar studies. The following table consists of pertinent variables, variable definition, and measurement.

Table 1: Variables Measured

Variable	Definition	Measurement
Synaptosome	• Synaptosomes are sphere-shaped synaptic boutons that are resealed in isotonic sucrose solution. • They are 0.5 to 1μm diameter in size. • Synaptosomes consist of presynaptic contents (synaptic vesicles, mitochondria, cytoskeleton proteins) and postsynaptic structures. • The pre- and postsynaptic structures reseal to form a 'snowman' figure. (Sokolow et al., 2012)	• Quantification of anti-SNAP25 polyclonal rabbit antibody will be conducted by Western blot and flow cytometry methods.
Aß	• Aß is a peptide capable of forming plaque aggregates. • In the monomeric form, Aß is	• Quantification of anti-10G4 polyclonal rabbit

	approximately 4.5kDa and 30- to 42-amino acid residues long.	antibody will be conducted via Western blot and flow cytometry. • 10G4 labels Aß plaques in AD brain but does not bind APP (Fein et al., 2008).
apoE (protein)	• ApoE is a 299-amino acid glycoprotein with a molecular weight of 34.2 kDa • ApoE is involved in cholesterol transport and is produced by astrocytes and microglia	• Quantification of anti-apoE (E6D7) mouse monoclonal antibody will be conducted via Western blot and flow cytometry analysis. • E6D7 reacts to amino acid on position 158 of apoE.
APOE (gene)	• The protein encoded by *APOE* is essential for the normal catabolism of triglyceride-rich lipoprotein constituents and transport of cholesterol in the brain. • *APOE* maps to chromosome 19.	• Samples were genotyped for *APOE* gene will exist in variations: *APOE 2/2, APOE 2/3, APOE 3/3, APOE 3/4, APOE 4/4, APOE 2/4*
Braak Stage	• Braak staging is a method of classifying NFT pathology in the brain. • Stage 1: no NFTs • Stage 2: NFTs are seen in entorhinal cortex, spreads towards neocortex • Stage 3: lesion density is increased extending into fusiform gyri, lingual gyri, hippocampus, amygdala • Stage 4: NFTs progress into temporal lobe and deeper cortical regions of CA3 and CA4 • Stage 5: tau lesions extend in a fan-like pattern in frontal and occipital cortex • Stage 6: NFTs reach occipital lobe into	• Braak stage will be graded by a neuropathologist through histopathology methods

	striatum	
Esterified cholesterol	• Esterified cholesterol is synthesized when an ester bond is formed between the carboxylate group of a fatty acid and the hydroxyl group of cholesterol. • Cholesteryl esters have a low solubility in water and are hydrophobic.	• Esterified cholesterol will be measured using fluorescent lipid stain Nile Red. • Nile Red binds to intracellular lipids.
Free cholesterol	• Free cholesterol is unesterified and synthesized in the endoplasmic reticulum of neurons and astrocytes. • Free cholesterol is found bound to cell membrane.	• Free cholesterol will be measured using fluorescent lipid stain Filipin. • Filipin binds to neuronal membrane cholesterol.
LDLR	• LDLR receptors function in the regulation of cholesterol in the brain. • LDLR interacts with apoE in an isoform dependent manner.	• Anti-LDL receptor monoclonal rabbit antibody reacts with amino acids 29-205 of human LDL receptor. • LDLR level will be measured with Western blot analysis
LRP1	• LRP1 receptor is a transmembrane protein expressed in neurons and glia.	• Anti-LRP1 receptor monoclonal rabbit antibody reacts with amino acid 4450 to the C-terminus of human LRP1 receptor. • LRP1 level will be measured with flow cytometry and Western blot analysis

3.3 Evaluating Existing Theories in Alzheimer's Disease

A middle range theory is a theory that explains a phenomenon that can be tested (Kim, 2000). There are several middle range theories pertinent to current AD research. These theories aim to explain genetic, molecular, and cellular alterations involved in AD pathogenesis. In nursing, it is important to analyze and evaluate theories to determine the direction of how to progress in research. Therefore, this chapter segment will examine competing middle range theories by giving a brief overview, historical underpinnings, analysis, and evaluation of each theory.

3.3.1 Amyloid Cascade Hypothesis

In the 1970's and 1980's many of the theories on AD pathology were disorganized and untestable. Such theories included the contribution of slow viruses, aluminum exposure, accelerating aging, and environmental toxins to AD pathogenesis. In the mid 1980's, Masters and colleagues (1985) had shown that plaques in AD were made of Aß peptide. Thereafter, geneticists hypothesized that chromosome 21 was responsible for the AD phenotype. In 1992, Hardy and Higgins provided evidence that AD is heterogenous and APP mutations are associated with early onset AD. This data made it possible to create transgenic mouse models of AD and led to the synthesis of the amyloid cascade hypothesis.

The amyloid cascade hypothesis implicates involvement of three genetic loci in early onset AD: 1) APP gene on chromosome 21, 2) presenilin 1 gene on chromosome 14, and 3) presenilin 2 gene on chromosome 1. Mutations in these genes alter amyloid precursor protein (APP) and mutated APP produces Aß 42 through the gamma secretase pathway. Gamma secretase cleavage is important because where the enzyme cleaves the protein determines the length of Aß. Aß that is cleaved at the 42 position, termed Aß42, is prone to aggregation and

accumulation. Aß42 is a pathological hallmark in AD and overproduction or inefficient clearance of Aß42 is thought to block neuronal transmission and contribute to degeneration of synapses that mediate memory and cognition (Hardy & Higgins, 1992; Reitz, 2012)

3.3.2 Tau Hypothesis

Tau is a microtubule-associated protein (MAP) that primarily provides stabilization to microtubules in axons (Morawe, Hiebel, Kern & Behl, 2012). The tau hypothesis in AD posits that abnormal post-translational alterations of tau lead to hyper-phosphorylation that changes tau conformation. This abnormal conformational change promotes tau aggregation resulting in NFT formation. Excessive NFT aggregation leads to microtubule instability and neuronal degeneration in AD (Morawe, Hiebel, Kern & Behl, 2012; Braak, Alafuzoff, Arzberger, Kretzschmar, & Del Tredeci, 2006). According the Braak (2006), NFTs initially accumulate in the entorhinal cortex and later progress to the hippocampus, limbic cortex, primary cortex, temporal lobe, and occipital lobe. Cognitive decline in AD is associated with limbic cortex NFT degeneration (Braak, Alafuzoff, Arzberger, Kretzschmar, & Del Tredeci, 2006).

3.3.3 Cholesterol Mechanisms in AD

In the 1990s, Sparks and colleagues observed that AD brain contained similar senile plaques to patients with coronary heart disease and found that feeding rabbits a diet high in cholesterol induced brain plaque formation (Sparks et al., 1990; Sparks et al., 1994). These initial observations led to wide spread research on the relationship of cholesterol and AD. However, evidence suggests that elevated serum and plasma cholesterol are not a risk factor in developing AD and serum cholesterol does not affect brain cholesterol homeostasis (Dietschy and Turley, 2004; Wood, Li, Muller & Eckert, 2014).

Most cholesterol in the brain is free unesterified cholesterol, which serves to maintain neurological functions such as neurotransmitter release, synaptic plasticity, and neuronal repair (Bjorkhem & Meaney, 2004). The BBB restricts large particles such as LDL from entering the brain and therefore, most cholesterol in the brain is synthesized by astrocytes and binds to apoE (Chen, Hui & Geiger, 2014). Currently, the structure of apoE-cholesterol complex in brain has not been established, but it is hypothesized to be a discoidal shape with phospholipids and unesterified cholesterol. This formulation is based on studies that have isolated lipoproteins from CSF (Chen, Hui & Geiger, 2014; De Chaves & Narayanaswami, 2008). After the synthesis of cholesterol-apoE complex, the complex is endocytosed into neurons through apoE receptors that are expressed by neurons such as LDLR, VLDLR, LRP1, and sorLA-1 (Holtzman, Herz & Bu, 2012). This is thought to contribute to Aß clearance through formation of apoE/cholesterol/Aß complex that subsequently carries soluble Aß to apoE receptors for internalization and degradation (Gylys et al., 2003; Arold et al., 2012; Tai et al., 2014). Therefore, cholesterol may be involved in Aß clearance from the synapse through its association with apoE.

3.4 Framework Structure

Due to the plethora of competing middle range theories in AD, a reformulation of theory is necessary. The reformulation of a theory must take into account all concepts of competing middle range theories that are deemed valid and pertinent to the research question. Although the amyloid cascade hypothesis holds a cogent argument in explaining the pathway to neurodegeneration in AD, there are several gaps in this theory.

Such gaps include an explanation of the role of apoE, apoE receptors, and cholesterol in synaptic Aß clearance (Longenberger & Shah, 2011; Nicholson, Methner & Ferreira, 2011).

Therefore, it is pertinent address how cholesterol, Aß, apoE receptors, and apoE levels change in normal, early, and late AD. It has been proposed that apoE and Aß bind and create an apoE-Aß complex (Gylys et al., 2003). This is thought to be important for soluble Aß clearance in the synapse through apoE receptors and changes in free cholesterol are involved (Arold et al., 2012). However, it is not known if significant changes in apoE receptor and cholesterol quantity levels impact clearance of Aß in synapses.

The working hypothesis is depicted in the figure below. First cholesterol and apoE in the brain is synthesized by astrocytes (Dietschy and Turley, 2004). ApoE is then lipidated by ABCA and an apoE-cholesterol complex is formed (Djelti et al., 2015; Vance, 2012). This complex is able to directly bind with Aß creating an apoE-cholesterol-Aß complex, which undergoes endocytosis by apoE receptors LDLR and LRP1 to deliver cholesterol to neurons (Holtzman, Herz & Bu, 2012) The region where Aß and apoE binding occurs is likely on the carboxy-terminal domain that overlaps the lipid binding region (Strittmatter et al., 1993; Tamamizu-Kato et al., 2008). Once cholesterol enters the neuron, unesterified cholesterol, also known as free cholesterol, is converted to cholesteryl-esters (esterified cholesterol) by enzymes ACAT1 and ACAT2 (Murphy et al., 2013; Djelti et al., 2015; Vance, 2012). Esterified cholesterol forms intracellular lipid droplets that are implicated to function in lipid storage. Esterified cholesterol can also be converted to 24S-hydroxycholesterol (24S-OH-C) that can cross the BBB (Murphy et al., 2013; Djelti et al., 2015; Vance, 2012).

Based on the framework, the working hypotheses are synaptic Aß clearance in late stage AD is dysfunctional related to: 1) a downregulation of LDLR and LRP1 that would normally internalize the HDL-cholesterol/apoE/Aß complex, 2) free cholesterol level changes in AD that impact how Aß is able to enter through the membrane, 3) esterified cholesterol level changes in

AD impacting cholesterol storage and potential membrane repair 4) *APOE* genotype-mediated differences in cholesterol content, that further contribute to impaired Aß clearance in AD.

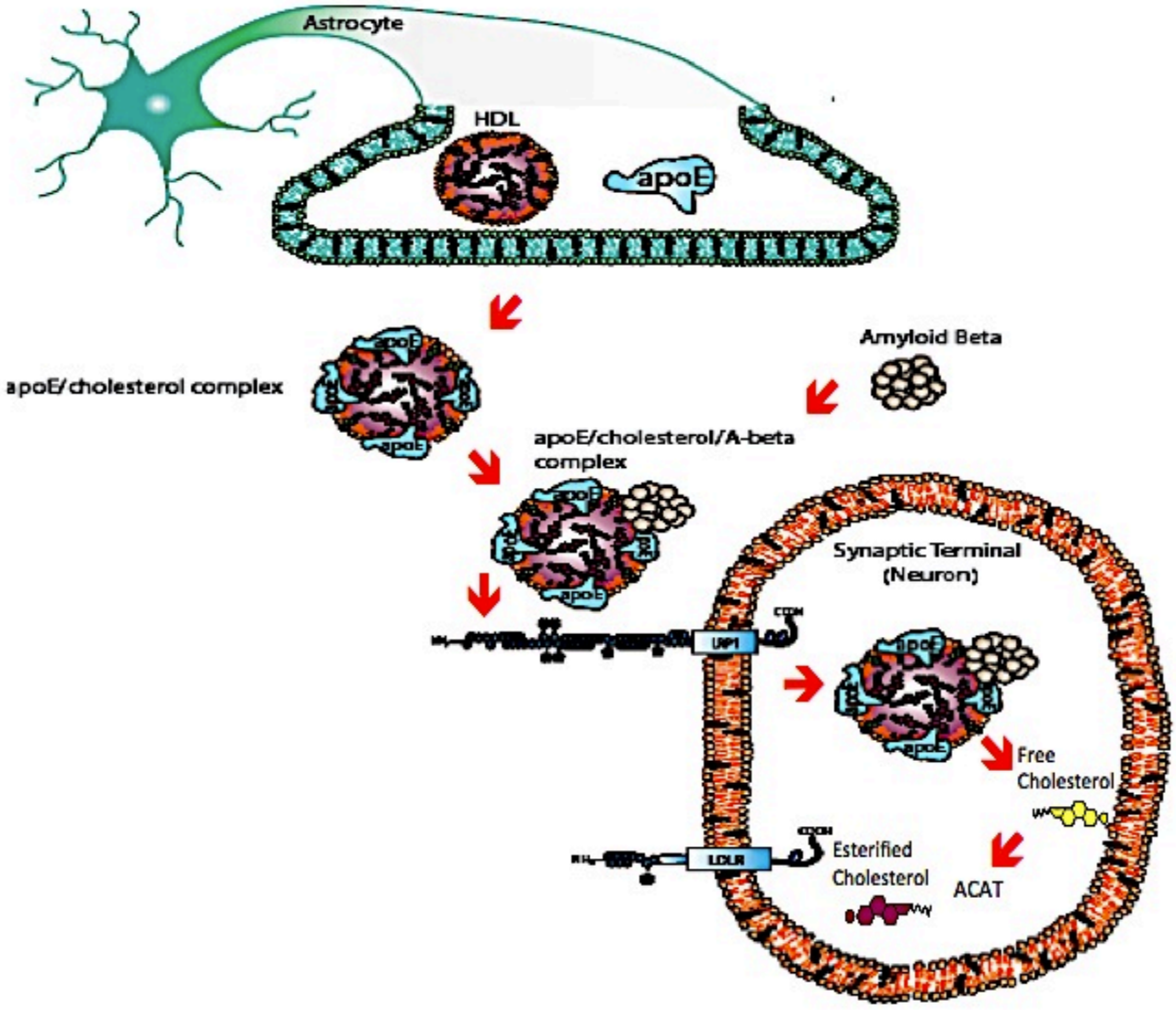


Figure 1. Synaptic Aß Clearance Mechanism
HDL cholesterol and apoE are manufactured *de novo* by astrocytes. ApoE is lipidated with HDL cholesterol by ABCA and is released from the astrocyte. The apoE-cholesterol complex binds Aß extracellularly and delivers Aß to LRP1 and LDLR for cellular internalization. After endocytosis, HDL is broken down into free cholesterol and is converted to esterified cholesterol by ACAT enzymes. Thereafter, esterified cholesterol forms intracellular lipid droplets sequestered intracellularly that are implicated to function in lipid storage. Esterified cholesterol can also be converted to 24S-hydroxycholesterol (24S-OH-C) that can cross the BBB to exit the brain.

Chapter 4

Methods

4.1 Samples

Human brain tissue from the University of California Los Angeles, University of Southern California, and University of California Irvine brain banks was used to complete the following experiments. Samples from human parietal cortex (areas 7, 39 and 40) with post-mortem intervals less than 12 hours were retrieved at autopsy from the University of California, Los Angeles, University of California Irvine, and the University of Southern California. Samples were obtained from a total of 52 cases (24 males, 28 females). Normal (n=13), early AD (n=11) and late stage AD (n=28) brain were used with a mean age of 88 years among samples (Table 3). All samples were minced and slowly frozen on the day of autopsy in 0.32M sucrose with protease inhibitors.

4.2 Setting

All experiments were conducted at the Gylys Lab of the University of California Los Angeles School of Nursing. The Janis V. Giorgi Flow Cytometry Core Facility at the University of California Los Angeles, Jonsson Comprehensive Cancer Center was utilized to conduct flow cytometry analysis on BD FACSCalibur and BD LSRII cytometers.

4.3 Study Procedures

4.3.1 P-2 Synaptosome preparation

Synaptosomes are isolated synaptic terminals from neurons that are resealed through homogenization of brain in isotonic sucrose (Gylys et al, 2004). Synaptosomes contain mitochondria, endosomes, and exocytotic apparatus. Synaptosome preparations enable

investigators to study synaptic components such as neurotransmitters and receptors *in vitro* (Gylys et al., 2004).

Unfixed samples were minced and slowly frozen the day of autopsy in 10% imethyl sulfoxide and 0.32 mol/L. Samples were then stored at −70°C until the day of homogenization. On the day of homogenization, samples were thawed and homogenized in 0.32 mol/L sucrose in 10 mmol/L Tris buffer (pH 7.4) with protease inhibitors: 2 mmol/L EGTA, 2 mmol/L EDTA, 0.2 mmol/L phenylmethyl sulfonyl fluoride, 4 μg/ml leupeptin, 4 μg/ml pepstatin, 5 μg/ml aprotinin, 20 μg/ml trypsin I. The tissue was homogenized in a glass homogenizer by eight up and down strokes at 800 rpm. The homogenate was spun at 1,000 g for ten minutes; the resulting supernatant was centrifuged at 10,000 g for 20 minutes to obtain the crude synaptosomal pellet (P-2). P-2 was resuspended in 0.32M sucrose with the P-2 (crude synaptosome) fraction in 0.32M sucrose solution with added chelating agents 0.5M EDTA and 0.2M EGTA; protease inhibitors 4 μg/ml pepstatin, 5 μg/ml aprotinin, 20 μg/ml trypsin inhibitor, 0.02M PMSF, 4 μg/ml leu-peptin, and buffer 1M TRIS pH 8.0 to remove nuclei and cell debris. The supernatant was centrifuged at 10,000*g* for 20 minutes resulting in a crude synaptosomal pellet (P-2). The final pellet was washed with 2 ml of phosphate-buffered saline (PBS) and centrifuged for 4 minutes at 2000*g* in a 4°C cold room. Aliquots of P-2 were cryopreserved in 0.32M sucrose and stored at -70°C until the day of the experiment (Gylys et al., 2004).

4.3.2 Flow Cytometry

Flow cytometry is a method of cell counting, cell sorting and biomarker detection. This is accomplished by suspending cells in a stream of fluid that passes a through a laser and electronic detection system. Lasers come in a variety of wavelengths and the use of filters separates the light coming from the cell and steers the photons towards a photon detector. As a cell passes

through the detectors of a flow cytometer, it scatters light and fluorophores on or inside the cell. Furthermore, the cell will absorb the incident light and emit photons in a range of wavelengths. The photons are detected and a current is generated proportional to the amount of incident light (Brown & Wittwer, 2000).

Table 2: Reagents and Antibodies

Variable Labeled	Antibody/ Stain	Manufacturer	Reactivity/ Isotype	Monoclonal/ Polyclonal
Aß	10G4	Cole Lab	Mouse IgG2b	Polyclonal
apoE	E6D7	Abcam	Mouse IgG1	Monoclonal
LDLR	Anti-LDLR	Abcam	Rabbit IgG	Monoclonal
LRP1	Anti-LRP1	Abcam	Rabbit IgG	Monoclonal
VGlut1	Anti-VGlut1	Abcam	Rabbit IgG	Monoclnal
Free Cholesterol	Filipin	Sigma-Aldrich	N/A	N/A
Esterified Cholesterol	Nile Red	Sigma-Aldrich	N/A	N/A
Zenon Alexa Fluor 488 Mouse IgG Labeling Kit	10G4, E6D7	Thermo Fisher Scientific	Mouse IgG	Monoclonal
Zenon Alexa Fluor 488 Rabbit Labeling Kit	vGlut	Thermo Fisher Scientific	Rabbit IgG	Monoclonal
Zenon Alexa Fluor Rabbit Labeling Kit	LDLR, LRP1	Thermo Fisher Scientific	Rabbit IgG	Monoclonal

P-2 aliquots were immunolabeled for Aß, LDLR, and LRP1 using antibodies against Aß (10G4), LDLR, and LRP1. 10G4 is directed against Aß residues 5-17, E6D7 is directed against apoE residue 158, anti-LDLR is directed against residues on the C-terminus of human LDLR, and anti-LRP1 is directed against residues on the C-terminus of LRP1. For the purposes of internal validation, vesicular glutamate 1 transporter (VGLUT1), a protein expressed in synaptic

vesicle membranes and plays a role in glutamate transport, was labeled with anti- VGLUT1 antibody direct against residues 150-250 of VGLUT1 and used as a negative control.

Filipin and Nile Red stains were used to label membrane cholesterol and esterified cholesterol respectively. Filipin is a fluorescent polyene antibiotic that has high affinity towards free (unesterified) cholesterol. The ultraviolet excitation of Filipin is approximately 360 nm with an emission of approximately 480 nm (Maxfield & Wüstner, 2012). Flow cytometry analysis of Filipin labeled was conducted on the BD LSRII cytometer through the DAPI channel, which is the path of emission on the ultraviolet laser through the 450/50 filter. Nile red dye labels esterified cholesterol within cells (Muller et al., 1984; ThermoFisher Scientific, 2016). Flow cytometry analysis of Nile Red labeled samples was conducted on the BD LSRII cytometer through the PE channel, which is the path of emission on the ultraviolet laser through the 575/25 filter.

Data was acquired using BD FCSCalibur and LSRII flow cytometers equipped with argon 488 nm, helium-neon 635 nm, and helium-cadmium 325 nm lasers. Debris were excluded by establishing a gating size threshold set on forward light scatter. For the purposes of the following experiments, a size gate was utilized to detect particles between 0.5-1.5 microns, which has been shown to detect a highly pure synaptosome prepration at approximately 95% (Gylys et al., 2004; Gylys et al., 2003). Analysis was performed using FCS Express software. There are two measurements that were obtained: RFU and % positives. RFU refers to relative fluorescence unit, which is a measurement of fluorophores on or in the cell passing through the laser beam emitting a signal relative to other cells. % positives refer to the % of cells in the population that possess the attribute being detected. Forward scatter is the light refracted in a

forward direction when a cell passes through the laser beam (large objects will refract more light than smaller objects leading to high forward scatter signals).

Flow cytometry provides accurate measurement of large populations of synaptosomes and is able to detect positive fraction/brightness of fluorescence, size, and particle complexity. This method also allows for sorting of particles when dual labeled to compare positive and negative particles of interest. It is important to note that flow cytometry measurements are relative and have no absolute value. Therefore, there must be controls initiated such as blank unstained sample as a negative control. Benefits of flow cytometry include the capability of separated cells into high purity samples and rapid computation of signals. Additionally, flow cytometry is able to detect multiple proteins in a single sample through dual labeling techniques. Limitations include relative quantification rather than absolute values and costly materials and reagents.

4.3.2.1 Immunolabeling of P-2 Fraction

P-2 aliquots were labeled according to a method for staining of intracellular antigens against Aß, LDLR, and LRP1. Pellets were fixed in 0.25% buffered paraformaldehyde for 1 hour in 4°C. Then samples were permeabilized in 0.2% Tween20 in PBS for 15 minutes at 37°C. Antibodies were labeled using Alexa Fluor 488 or 647 reagents and incubated at room temperature for 30 minutes. Samples were then washed two times with 1 ml 0.2% Tween20 in PBS and resuspended in PBS buffer (Henkins et al., 2012; Gylys et al., 2004; Schmid, Uittenbogaart & Giorgi, 1991).

4.3.2.2 Lipid Staining

P-2 aliquots were labeled with Filipin, a fluorescent polyene antibiotic that binds unesterified ‘free’ cholesterol. The ultraviolet excitation of Filipin is approximately 360 nm with

an emission of approximately 480 nm (Maxfield & Wüstner, 2012). Nile red (5H-Benzo[α]phenoxazin-5-one, 9-(diethylamino)- 7385-67-3), which is a lipid stain that is able to localize neutral lipid droplets within cells, was used to label esterified cholesterol (Muller et al., 1984; ThermoFisher Scientific, 2016). The approximate excitation of Nile Red is 552 nm with an emission of approximately 636 nm in methanol or DMSO (ThermoFisher Scientific, 2016). Pellets were fixed in 0.25% buffered paraformaldehyde for 1 hour in 4°C. Then samples were washed two times with 1 ml PBS and resuspended in PBS buffer. Samples and labeling stains were protected from light during experiments (Arold et al., 2012; Gylys et al., 2004; Schmid, Uittenbogaart & Giorgi, 1991).

4.3.2.3 Western Blot

Using the synaptosome preparation, Western blot analysis was conducted to identify specific proteins in a synaptosome samples. The Western blot technique uses five elements: 1) sample preparation, 2) separation of proteins by size through gel electrophoresis, 3) transferring to a membrane, 4) Marking target protein using primary and secondary antibody to visualize, and 5) analysis and quantification of signal density with UVP software. The protein concentration of brain synaptosome preparation was determined using BCA assays. Boiled samples were electrophoresed on 4-20% SDS-page gels. Membranes were blocked for 1 hour at room temperature in 5% BSA, followed by incubation overnight at 4°C with the primary antibodies in PBS containing 0.01% Tween. Membranes were incubated for 1 h with anti-rabbit IgG horseradish peroxidase-conjugate. Membranes were incbated with SuperSignal West Dura substrate and exposed to an OptiChemi HR Camera 600. The benefits of this technique include high sensitivity and specificity, low cost, and the ability to measure the size of proteins. Limitations of Western blot are that the technique is not able to detect more than one type of

protein on one gel and there may also be some non-specific binding depending on antibody propoerties.

4.4 Data Analysis

Analysis of flow cytometry data was performed using FCS Express software (DeNovo Software, Ontario, Canada) while Western blot signals were quantified and analyzed with Biospectrum VisionWorks®2D Analysis Software (UVP, Upland, California). Data were analyzed using SPSS Statistical software (IBM) for statistical computation. Two-tailed, unpaired student t-test to test for mean differences ± standard error of the mean (SEM) in Aß in LDLR- and LRP1-positive synapses, apoE in LDLR- and LRP1-positive synapses, VGLUT1- in LDLR- and LRP1-positive synapses, and LRP1 levels in normal synaptosomes versus late AD synaptosomes. Analysis of variance (ANOVA) with post-hoc Tukey test was used to describe differences in LDLR, esterified cholesterol, and free cholesterol levels across *APOE* genotype and AD stage.

Chapter 5

Results

5.1 Introduction

As detailed in previous chapters, the main objective of the current study is to elucidate dysfunctional mechanisms in Aß clearance in AD, which remains unclear. To address this research problem, associations of apoE receptors and cholesterol levels in AD versus normals were investigated. This chapter presents the findings of the study experiments, which aimed to: 1) determine associations between apoE receptors, apoE, and Aß in AD synaptic pathology, 2) quantify LDLR and LRP1 levels in human synapses of normal versus early and late AD to determine associations of receptor level and AD progression, 3) determine the relationship between esterified cholesterol and free cholesterol levels in AD pathogenesis and 4) determine the association between esterified cholesterol and free cholesterol levels in relation to *APOE* genotype.

5.2 Aim 1: ApoE receptor associations with apoE and Aß in AD synaptic pathology

5.2.1 Aß and apoE are increased in LDLR- and LRP1-positive synapses in late AD

Aß % positives labeling was markedly increased in both LDLR-positive synaptosomes (92.52±2.01, $p<0.00$) and LRP1-positive synaptosomes (94.06%±0.80, $p<0.00$) compared to broader synaptosome population (60.63%±5.73; Figure 2.1A-C, Figure 2.3). Likewise, apoE % positive labeling was also dramatically increased in LDLR-positive synaptosomes (92.52%±2.01,$p<0.00$) and LRP1-positive synaptosomes (94.06%±0.80, $p<0.00$) compared to larger synaptosome population (12.30%±4.75; Figure 2.2A-C, Figure 2.3). VGLUT1 % positive labeling, a negative control, did not was similar in LDLR-positive synaptosomes (71.83%±2.50) and LRP1-positive synaptosomes (56.33%±2.80; Figure 2.3) relative to the entire synaptsome

population (50.52%±8.55; Figure 2.3). The RFU data show that Aß is increased in LDLR- and LRP1- positive synapses (RFU=304.46±32.36, RFU=299.24±38.81) compared to total Aß in late AD synaptosomes (RFU=182.61±21.60, p=0.011, p=0.025). However, there were no significant differences in total apoE versus apoE RFU in LDLR- and LRP1-positive synapses in late AD synaptosomes (p=0.755, p=0.129; Figure 2.3).

5.3 Aim 2: ApoE receptor associations with AD synaptic pathology

5.3.1 LDLR is increased in late AD versus normal synapses

The previous experiment implies that Aß and apoE are enriched in LDLR- and LRP1-positive synapses in late stage AD. Therefore, the next experiments examined differences in receptor levels across normal (n=3), early AD (n=7), and late AD (n=4) brain. Western blot analysis showed significant increases in LDLR mean density in late AD (Mean Density=214.39±16.34) versus normal samples (Mean Density=63.44±SEM=1.29, $p<0.000$, F=9.01; Figure 3A-B). Flow cytometry was not conducted due to unavailability of anti-LDLR antibody from the manufacturer.

5.3.2 LRP1 is reduced in late AD versus normal synapses

Because the downregulation of apoE receptors may be responsible for dysfunctional clearance in AD, human synaptosomes from parietal cortex were labeled for LRP1 with anti-LRP1 antibody. Flow cytometry analysis and Western blot were utilized for analysis and internal validation. Results of flow cytometry analysis showed a significant decrease in relative fluorescence of LRP1 in late AD samples (n=22, RFU=24.60±0.92) compared to normal samples (n=13, RFU=29.90±2.17, p=0.038; Figure 4.2A-C). Western blot analysis concurred with the flow cytometry results, showing significantly reduced mean density of LRP1 in late AD

synaptosomes (n=11, Mean Density=140.46±34.94) compared to normals (n=3, Mean Density=459.37±48.43, p=0.0045; Figure 4.1A-B).

5.4 Aim 3: Cholesterol levels and association with AD synaptic pathology

5.4.1 Free cholesterol is increased in AD versus normal synapses

According to several lines of evidence, membrane dysfunction is implicated in AD and free cholesterol level alterations in the membrane may contribute to AD pathology (Rushworth & Hooper, 2011; Simmons & Gerl, 2010). Results of the flow cytometry analysis showed an upward trend of free cholesterol labeling with increased Braak stage. There was a significant increase in the fraction of synaptosomes positive for free cholesterol (% positive) in late AD (n=13, 98.51%±0.247) compared to normal controls (n=27, 96.91±0.612, p=0.034, F=3.64; Figure 5.1A-D). However, no significant AD stage changes were noted in RFU data (p=0.170; Figure 5.1E).

5.4.2 Esterified cholesterol is reduced in AD versus normal synapses

Unlike free cholesterol, esterified cholesterol is not found in the cell membrane, but is found intracellularly, sequestered as lipid droplets (Puglielli et al., 2001; Bryleva et al., 2010; Murphy et al., 2013). Intracellular lipid droplets are thought to function in lipid storage (Bryleva et al., 2010; Murphy et al., 2013). Results of the flow cytometry analysis of synaptosomes labeled for esterified cholesterol (Nile Red) showed a downward trend associated with increased Braak stage. Esterified cholesterol was significantly reduced in late AD (RFU=3617.78±136.12) compared to normal controls (RFU=4615.47±208.63, p<0.000, F=9.41; Figure 5.2A-D). However, there were no significant changes in % positives (p=0.499, Figure 5.2E)

5.5 Aim 4: Determine variations in esterified cholesterol and free cholesterol levels in relation to *APOE* genotype and AD stage

5.5.1 Free cholesterol and esterified cholesterol are not altered across *APOE* genotype

Because apoE is the primary cholesterol carrier in the brain, esterified cholesterol and free cholesterol levels across *APOE* genotype were measured. There were no significant differences in free cholesterol level (Filipin) ($p=0.122$) or esterified cholesterol (Nile Red) ($p=0.170$) between *APOE 3/3* and *APOE +e4* synaptosomes.

5.5.2 Free cholesterol is significantly increased in late AD among *APOE 3/3* synaptosomes

In previous experiments, cholesterol levels were analyzed over AD disease stage. Because *APOE* variations are thought to be the greatest genetic risk factor for developing sporadic AD, a secondary analysis of free cholesterol over *APOE* genotype was conducted. Results of flow cytometry analysis revealed that free cholesterol (Filipin) is increased in late AD (RFU=17057.89±816.11) compared to normals among *APOE 3/3* synaptosomes (RFU=20553.59±960.05, $p=0.021$, $F=4.632$; Figure 6.1).

5.5.3 Esterified cholesterol is reduced in late AD among *APOE 3/3* synaptosomes

Unlike free cholesterol, esterified cholesterol (Nile Red) saw a reduced trend across increasing AD disease stage. Results of flow cytometry analysis revealed that esterified cholesterol (Nile Red) is reduced in late AD (RFU=4543.01±260.73) compared to normals among *APOE 3/3* synaptosomes (RFU=3648.49±179.39, $p=0.023$, $F=4.509$)(Figure 6.2).

5.5.4 Cholesterol levels are not significantly altered in early AD versus late AD among *APOE +e4* synaptosomes

In addition to *APOE 3/3,* which is the most prevalent variant of the *APOE* gene, *APOE 3/4* and *APOE 4/4* were analyzed across disease stage. For the purposes of this experiment, *APOE 3/4* and *APOE 4/4* were grouped together. Results of flow cytometry analysis revealed that there are no significant changes in esterified cholesterol % positives (p=0.489) and RFU (p=0.066) (Figure 7.2). Similarly, there are no significant changes in free cholesterol % positivs (p=0.819) and RFU (p=0.457) across disease stage among *APOE +e4* synaptosomes (Figure 7.1). These results are in contrast to previous secondary analysis of cholesterol levels across disease stage in *APOE* 3/3 (Figure 6.3-6.4).

Chapter 6

Discussion and Conclusions

6.1 Discussion

Dysfunction of Aß clearance from synapses is hypothesized to contribute to synaptic loss, which occurs in the early stages of AD (Gylys et al., 2004; Shinohara, Petersen, Dickson & Bu, 2013; Thinakaran & Koo, 2008). Multiple lines of evidence have implied that associations between Aß, apoE, apoE receptors, and cholesterol play a role in dysfunctional Aß clearance in AD pathology (Gylys et al., 2007; Arold et al., 2012; Bien-Ly et al., 2011). However, the exact mechanisms and pathways remain unclear. The results of the experiments described in the previous chapter reveal several possible mechanisms and contribute to the growing body of knowledge regarding dysfunctional Aß clearance in AD synapses. The purpose of this chapter is to discuss implications of the experimental results from the previous chapter and address the necessity for future research in Aß clearance pathways.

6.1.1 Aß and apoE co-localize with LDLR and LRP1 in synapses

In the initial experiment, Aß and apoE were dual-labeled with LDLR and LRP1 in synaptosome preparations of late AD parietal cortex. This experiment demonstrated that Aß and apoE are enriched in LDLR- and LRP1-positive late AD synapses compared to total Aß and apoE levels based on % positive levels. RFU data also show that Aß is enriched in LDLR- and LRP1-positive synapses in late AD. However, there were not differences detected in RFU of total apoE versus apoE in LDLR- and LRP1-positive synapses in late AD. Because only late AD cases were utilized for the experiment, the lack of significant changes in RFU level of apoE versus apoE in LDLR- and LRP1-positives reflect reduced apoE level within AD synapses contributing to lack of Aß clearance in AD pathology. Results are consistent with the theory that

Aß and apoE form an apoE/Aß complex, with apoE and amyloid-β (Aβ) peptide binding occuring at residues 244–272 of apoE and residues 12–28 of Aβ (Liu et al., 2011; Sadowski et al., 2006). The apoE/Aß complex is hypothesized to promote Aß clearance in the synapse, possibly through interaction with LDLR and LRP1 receptors (Gylys et al, 2003; Tai et al., 2013; Arold et al., 2012).

Early experiments by Gylys and colleagues (2003) found that treating synaptosomes with soluble Aß and apoE increases Aß-positive synapses, demonstrating internalization of Aß in the synapse. Furthermore, when RAP, an LRP1 inhibitor, was applied to treated cells, Aß-positive synapses decreased. This is consistent with LRP1 functioning in the internalization of Aß from the synapse (Gylys et al., 2003). Concurrently, a study by Shibata et al. (2000) showed that knock-out mice of LRP1 and LDLR reduced Aß clearance, implying that apoE receptors (LRP1 and LDLR) are involved in Aß clearance.

Although a number of studies have shown evidence of an apoE/Aβ complex, Verghese and colleagues (2013) found that utilizing physiologically relevant ratios of Aβ:apoE in human CSF and plasma brain homogenates from humans and FAD-Tg mice produced minimal amounts of apoE/Aβ complex (Verghese et al. 2013). These investigators suggest that apoE and Aβ do not directly interact with each other, but rather, compete for LRP1 binding sites. This may account for the perceived apoE and Aβ association (Verghese et al., 2013).

In response to the results of Vergehese and colleagues (2013), Tai and colleagues (2014) argued that concentration of Aß is significantly lower than apoE in CSF, plasma and brain homogenates from humans. Furthermore, Tai and colleagues (2014) pointed out that the method of using density gradient centrifugation may have further reduced apoE, and impacted the apoE/Aβ complex structure in the experiments conducted by Vergehese and colleagues (2013).

Overall, the theory of apoE and Aß forming a complex that is cleared from the synapse by LDLR and LRP1 is controversial. However, the results of this current experiment showing an enrichment in Aß and apoE in LDLR- and LRP1-positive synapse concur with the apoE/Aß complex theory in Aß clearance, suggesting that apoE and Aß are cleared from the synapse via LDLR and LRP1.

6.1.2 ApoE receptor level alterations are prominent in late stage AD

6.1.2.1 LRP1 receptor is downregulated in AD and contributes to reduced Aß clearance

Because the previous experiment supports the theory that LDLR and LRP1 clear the apoE/Aß complex from synapses in late AD, the second set of experiments aimed to address if the severity of AD pathogenesis, as measured by Braak stage, is associated with apoE receptor level differences. The working hypothesis was that both LDLR and LRP1 would be reduced in late AD compared to normal synaptosomes. Such differences in receptor levels across Braak stage might then be responsible for dysfunction in Aß clearance in late AD.

Utilizing flow cytometry and Western blot analysis, LRP1 receptor levels were measured in late stage AD versus normal synaptosomes. Both Western blot and flow cytometry demonstrated that LRP1 was reduced in late stage AD versus normal synaptosomes. This result is consistent with the working hypothesis and AD stage-associated reduction in LRP1 reflected in the literature.

Kang and colleagues (2000) found that increased LRP1 expression is associated with later onset of pathogenesis in individuals with AD through analysis of retrospective clinical data (Kang et al., 2000). To further explore why increased LRP1 levels may prevent early onset AD, Li and colleagues (2001) hypothesized that there are differences in apoE receptor efficiencies.

Investigators conducted experiments testing endocytotic rates among apoE receptors. In these experiments, LRP1 was found to be highly efficient and endocytosed ligands at higher rates compared to other members of the LDLR family (Li, Lu, Marzolo & Bu, 2001). Structurally, LRP1 differs from other LDLR family receptors containing a Y*XX*L motif that provides a prominent cellular signal for endocytosis (Li, Lu, Marzolo & Bu, 2001). LRP1 also undergoes receptor endocytosis and recycling with or without the presence of a ligand. This may be due to the multiple NP*X*Y and the Y*XX*L signaling structures located on the cytoplasmic tail (Li, Lu, Marzolo & Bu, 2001). Therefore, LRP's efficiency in facilitating Aβ clearance through endocytic pathways likely involves signaling structures in its cytoplasmic tail.

Kanekiyo and colleagues (2013) conducted experiments using *in vitro* and *in vivo* models focusing on neuronal Aß clearance related to LRP1. In these experiments, confocal microscopy and immunohistochemical imaging of the neurons from the forebrain of LRP1 knock-out mice demonstrated that LRP1 deletion suppresses Aß clearance (Kanekiyo et al., 2013). Additionally, Tanokashira and colleagues (2015) conducted experiments on rat primary neurons and demonstrated that LRP1 and BACE 1 interact with each other. In this study, downregulation in LRP1 was seen to increase BACE1 expression and Aß levels (Tanokashira et al., 2015).

This evidence is consistent with the results of the current experiment and suggests that the downregulation of LRP1 may be responsible for reduced Aß clearance from the synapse in late AD. Therefore, LRP1 upregulation may have therapeutic functions that prevent against AD by facilitating Aß clearance. Future studies need to evaluate these hypotheses further and explore reasons why LRP1 is downregulated in late AD. This may be done through conducting functional studies that investigate LRP1 structural binding capacity to Aß, transcription of LRP1, LRP1 trafficking, LRP1 recycling, and dysfunction in LRP1 production.

6.1.2.2 LDLR is upregulated in AD synapses and may be compensating for Aß clearance dysfunction

Regarding LDLR, Western blot results demonstrate that there are significant increases in mean density of LDLR across Braak Stage, where LDLR level was highest at the late AD stage. However, this result is inconsistent with multiple lines of evidence and the original hypothesis of the experiment, which predicted LDLR downregulation in late AD.

De Oliveira and colleagues (2014) found that LDLR knockout (LDLR -/-) mice that lack LDLR, regardless of Aß treatment, demonstrated significant memory impairment. Additionally, LDLR knockout mice treated with Aß1-40 peptide showed increased hippocampal gliosis and Aß induced neurotoxicity (De Oliveira et al., 2014). Additionally, Kim and colleagues (2009) found that upregulation of LDLR in primary neuronal culture and APP transgenic mice increased Aß clearance. Overall, several lines of evidence suggest that LDLR facilitates Aß clearance. This is inconsistent with the results of the current experiment where there was an increase in LDLR in late AD.

This unexpected increase in LDLR in late AD may be attributed to LDLR affinity towards apoE. Studies demonstrate that LDLR binds to apoE in an isoform-dependent manner and is the only member of the LDLR family with preference for apoE isoform binding (Shu et al., 2014). The apoE4 isoform binds to LDLR at higher affinities compared to apoE3 and apoE2 (Johnson et al., 2014; Cheng et al., 2005). ApoE4 is also associated with poor lipid and Aß binding, which may contribute to reduced Aß clearance (Bien-Ly et al., 2011; Cheng et al., 2005). In the current experiment, all late AD samples had an E4 allele. Therefore, the results may be reflective of poor lipid binding capacity of apoE4 and reduced Aß clearance. Thus, the *APOE e4* allele may have played a role as a mediating variable in increasing LDLR levels in this

experiment, which is well-established in the literature (Bachmeier et al., 2013; Bien-Ly et al., 2011; Cheng et al., 2005).

Another interpretation of these results may be attributed to a compensatory mechanism to upregulate LDLR in late AD to facilitate Aß clearance. Experiments by Abisamba and colleagues (2010) utilized cultured primary neurons that overexpressed APP and subsequently increased Aß levels. Investigators saw increased LDLR levels in the samples with increased Aß. According to Abisamba and colleagues, this may be a compensatory response for reduced intracellular sterols, which leads to increased LDLR transcription (Abisamba et al., 2010). To confirm this hypothesis, however, more functional studies in LDLR production, trafficking, and recycling in AD synapses versus normal controls need to be conducted. Moreover, experiments that investigate cholesterol associations with AD pathogenesis need to be conducted since reduced intracellular sterols may impact LDLR transcription (Abisamba et al., 2010).

6.1.3 Cholesterol level alterations are prominent in late stage AD

6.1.3.1 Increase in free cholesterol level is associated with AD pathology

Cholesterol pool concentrations in the brain are thought to play an important role in AD pathogenesis (Petrov, Kasimov & Zefirov, 2016). Cholesterol in the brain exists in two major pools: the unesterified form (free cholesterol), which accounts for 95% of cholesterol in the brain, and the esterified form, which constitutes 5% of total cholesterol. Free cholesterol is found in cell membranes and is thought to function in cellular membrane fluidity, electrical transduction, structure, repair, and permeability (Dietschy, 2009; Petrov, Kasimov & Zefirov, 2016). HDL, which contains free cholesterol and esterified cholesterol, is produced by astrocytes and binds to apoE via lipidation by ABC transporters. It is subsequently transported to neurons by apoE for the purposes of membrane repair and cellular maintenance (Dietschy, 2009). The previous

experiment in this paper supports the theory that apoE particles bind to Aß and are internalized by LDLR and LRP1 to promote Aß clearance from the synapse (Gylys et al, 2003; Tai et al., 2013; Arold et al., 2012). Because of the potential role of cholesterol in apoE lipidation and apoE/Aß complex formation (Gylys et al., 2003; Gylys et al., 2007; Arold et al., 2012), the next experiments investigated cholesterol pool associations across AD stage. These experiments demonstrated significant increases in free cholesterol in late AD synapses versus normal synapses. This is consistent with the existing literature on free cholesterol in AD.

Multiple lines of evidence suggest that membrane-bound cholesterol (free cholesterol) is associated with AD pathogenesis. Schneider and colleagues (2006) conducted experiments in neuronal cultures showing that reduction in free cholesterol is associated with reduction in Aß. Furthermore, when free cholesterol was increased, Aß aggregation levels also increased (Schneider, Schulz-Schaeffer, Hartmann, Schulz & Simons, 2006). Other results from Ferrara and colleagues (2008) used human neuroblastoma cells and a viability cell assay to determine the impact of free cholesterol on Aß neurotoxicity. They found that free cholesterol promotes Aß neurotoxicity and is associated with a reduction of cell viability markers. Furthermore, Abramov and colleagues (2011) used hippocampal neuronal cultures to demonstrate that increasing membrane cholesterol caused neuronal death when treated with Aß.

Other lines of evidence implicate lipid rafts in the increase in membrane cholesterol levels, which contributes to AD pathology (Pike, 2006; Simons & Gerl, 2010; Rushworth & Hooper, 2011). Lipid rafts are composed of free cholesterol and GM1 ganglioside that is thought to increase ß-secretase cleavage of APP, producing the malignant Aß42, which is prone to aggregation (Gylys et al., 2007). Gylys and colleagues (2007) dual labeled synaptosomes with Aß, free cholesterol, and GM1 ganglioside markers. There were significant increases in free

cholesterol and GM1 ganglioside synapses in Aß-positive terminals in AD (Gylys et al., 2007). Concurrent with these results, recent experiments by Djelti and colleagues (2015) isolated lipid rafts from mouse hippocampi on sucrose gradients and found a significant increase in free cholesterol content, increased APP, and increased Aß peptide levels. The increased cholesterol was accompanied by endosomal compartment enlargement that favors APP recycling from the cell surface (Djelti et al., 2015). In addition to increasing Aß production, several lines of evidence also suggest that increased lipid rafts in the cellular membrane obstruct trafficking of proteins through the membrane which may lead to reduced Aß clearance and synaptic function in AD (Hicks, Nalivaeva & Turner, 2012).

The observed increases in free cholesterol in late AD synapses versus normal synapses is in agreement with the current literature on lipid rafts stimulating APP towards the amyloidogenic pathway and increased production of Aß (Djelti et al., 2015; Gylys et al., 2007), and with several lines of evidence implicating increasing levels of free cholesterol contributing to increased Aß neurotoxicity in AD (Pike, 2006; Simons & Gerl, 2010; Rushworth & Hooper, 2011; Schulz-Schaeffer, Hartmann, Schulz & Simons, 2006).

6.1.3.2 Increase in esterified cholesterol level is associated with synaptic health

Historically, much of the literature has focused on free cholesterol due to its abundance in the brain. However, recent literature focuses on cholesterol turnover and conversion in the brain contributing to AD pathogenesis (Murphy et al., 2013). To investigate differences in cholesterol pool dynamics in AD, esterified cholesterol was labeled and measured across AD stage. Esterified cholesterol is sequestered intracellularly as lipid droplets in neurons and is thought to function as cholesterol storage in the brain (Murphy et al., 2013; Bryleva et al., 2010). The

present work demonstrates that esterified cholesterol levels, unlike free cholesterol levels, are reduced in late AD synapses compared to normal synapses. These results may implicate esterified cholesterol's role in lipid storage and homeostasis to maintain synaptic health. However, the literature measuring esterified cholesterol in AD is scant and inconsistent.

Liu, Peterson and Schubert (1998) labeled esterified cholesterol and free cholesterol in rat neuronal cell cultures to investigate associations between neurotoxic Aß fibrils, intracellular vesicular trafficking, and cholesterol homeostasis. Experimental results demonstrated that neurotoxic Aß fibril treatment was associated with increased free cholesterol content in the membrane, reduced esterified cholesterol levels, and an increase in a vesicular exocytotic marker. Furthermore, these observations were seen rapidly, within 3h of Aß treatment, prior to cell death caused by Aß. This suggests that Aß fibrils alter cholesterol homeostasis through vesicular trafficking mechanisms and that free cholesterol may regulate vesicular trafficking that are involved in cellular cholesterol homeostasis. Alternatively, Aβ fibrils may bind to free cholesterol in the membrane and attenuate the conversion of free cholesterol to esterified cholesterol (Liu, Peterson and Schubert, 1998). These data are consistent with the current experimental results in this chapter.

More recent studies, however, conflict with the current experimental results. A study conducted by Hutter-Paier and colleagues (2004) inhibited ACAT in transgenic mice expressing human APP containing the London and Swedish mutations using ACAT inhibitor, CP113,818. Results showed that ACAT inhibition reduced esterified cholesterol levels by 89%, reduced accumulation of Aß plaques by 88%-99%, reduced insoluble Aß by 83%-96%, and reduced soluble Aß by 34% in brain homogenates. There was no significant change in free cholesterol level since free cholesterol is not impacted by ACAT activity and is immobilized in myelin

(Hutter-Paier et al., 2004). Behaviorally, spatial learning and memory in mice were improved and correlated with reduced Aß levels suggesting that reduced esterified cholesterol through ACAT inhibition may associated with reducing Aß (Hutter-Paier et al., 2004). Similarly, studies by Bryleva and colleagues (2010) and Shibuya and colleagues (2015) demonstrated that ACAT1 deficient mice (ACAT -/-) resulted in reduced APP (human APP with Swedish mutation) levels and Aß levels (Bryleva et al., 2010). These studies imply that reduced levels of esterified cholesterol prevent Aß neurotoxicity in AD, which is inconsistent with the current experimental results. However, studies regarding esterified cholesterol and AD remain scant with small sample sizes in animal models rather than human models. Mechanisms regarding the molecular relationships between esterified cholesterol and Aß levels remain unclear.

The literature regarding esterified cholesterol in AD is controversial. Experimental results vary among studies and methods in measuring esterified cholesterol are inconsistent. Because cholesterol pool dynamics associated with AD pathogenesis remain unclear and difficult to measure due to fast turnover of cholesterol pools, more research is needed to investigate the potential mechanisms underlying cholesterol esterification. Functional studies and imaging studies in total cholesterol versus esterified and free cholesterol turnover need to be conducted in human models.

6.1.4 *APOE 3/3* is associated with dynamic cholesterol pool alterations while *APOE e4* is associated with static cholesterol pool levels across AD stage

Because the previous experiments suggested that Aß clearance in AD is dysfunctional, perhaps due to altered cholesterol dynamics, the next experiments aimed to elucidate underlying *APOE* genotypical associations with cholesterol levels in AD. These experiments demonstrated significant increases in free cholesterol and reduced esterified cholesterol levels in late AD

compared to normal synaptosomes within the *APOE 3/3* cohort. In contrast, there were no differences in free cholesterol and esterified cholesterol levels across AD stage among samples containing the *APOE e4* allele. These results are consistent with the literature and imply that dynamic shifts in cholesterol pools are necessary in maintaining synaptic function and response to Aß toxicity, which may be impaired with limited lipidation capacity of the apoE4 isoform. Furthermore, there were no significant differences in esterified cholesterol levels and free cholesterol levels between *APOE 3/3* and *APOE +e4* synaptosomes.

Arold and colleagues (2012) utilized synaptosome preparations from postmortem human neuronal tissue and apoE-TR mice to measure free cholesterol across apoE isoform. Consistent with the Aß clearance role of apoE, apoE level was higher in Aβ-positive synapses in normal samples compared to late AD samples. Furthermore, the *APOE e2* allele expressed higher levels of apoE compared to *e4* samples and this elevated apoE was accompanied by elevated free cholesterol. These results suggest that the higher lipidation capacity of apoE2 is optimal for Aß clearance compared to apoE4 (Arold et al., 2012). Furthermore, Hu and colleagues (2015) conducted a study in apoE-TR mice that expressed human apoE4. Results demonstrated that mice expressing apoE4 had reduced apoE-associated cholesterol and increased Aß accumulation compared to mice expressing apoE3 and apoE2 isoforms. In agreement with these results, Sinohara and colleagues (2015) used retrospective clinical data to show that the *APOE e2* allele was associated with reduced cognitive decline during aging. Investigators also validated evidence with experiments on aged apoE2-TR mice that exhibited reduced synaptic loss and increased levels of apoE compared to apoE3-TR and apoE4-TR mice. Additionally, free cholesterol was reduced among *APOE e2* mice, but not in *APOE e4* mice (Sinohara et al., 2015).

These results suggest that *APOE 3/3* genotype mediates cholesterol levels across AD stage. However, the *APOE +e4* does not show this characteristic. This is consistent with apoE4's poor lipidation capacity compared to other isoforms, which is thought to contribute to dysfunctional Aß clearance (Arold et al., 2012, Sinohara et al., 2015, Hu et al, 2015). Overall, the current experimental results suggest that dynamic cholesterol pools may contribute to synaptic function.

6.2 Conclusions and Implications for Future Research

This body of work examines alterations of apoE receptors and cholesterol levels in human cortical synapses. The main aims were to elucidate relationships between apoE receptors and cholesterol in AD synapses. Synaptic dysfunction and loss are early occurrences in AD pathology and it is imperative to elucidate the mechanisms involved. The results of these experiments contribute to the growing body of literature regarding potential pathways of dysfunctional synaptic Aß clearance in AD and demonstrate significant associations between LDLR, LRP1, Aß, apoE, and cholesterol levels in synaptic dysfunction across AD stage and *APOE* genotype.

The current experimental results, in concurrence with current conclusions in the literature, support the hypothesis that Aß and apoE co-localize with synaptic LDLR and LRP1 receptors in late AD. These data may indicate that apoE and Aß complex and are uptaken into apoE receptors (LDLR and LRP1) facilitating Aß clearance from the synapse. Furthermore, results support the role of LRP1 in being the more efficient receptor among LDLR family receptors for Aß clearance in the synapse and that reduction in LRP1 levels seen late in AD causes dysfunction in Aß clearance. LDLR, on the other hand, has a high affinity for apoE4 and may be a less efficient and compensatory receptor in Aß clearance from the synapse in late AD.

Results also indicate that cholesterol pool dynamics may contribute to dysfunctional Aß clearance from the membrane. Free cholesterol is increased in late AD and may contribute to Aß clearance dysfunction through obstructing membrane plasticity, intracellular diffusion of proteins, or increase Aß through lipid raft and APP interactions. Esterified cholesterol reduction may be pathological in AD since the reduction seen in AD may signify lipid storage depletion. Aβ fibrils also may bind to free cholesterol in the membrane and attenuate the conversion of free cholesterol to esterified cholesterol and deplete esterified cholesterol. Lastly, this body of research suggests differences in cholesterol dynamics with apoE3 isoforms. Overall, this data suggest *APOE* genotype across AD stage is associated with cholesterol pool dynamics and apoE receptor level differences, which may be associated with the degree of Aß clearance through apoE in the synapse.

Future research needs to focus on confirming these associations through functional studies in synapses. Functional studies may include examination on how *APOE* genotype and Aß/apoE level impact synaptic membrane potential, mitochondrial function, and chemical LTP. Studies must include larger sample sizes across Braak stage and *APOE* genotype, labeling of apoE receptors, and cholesterol labeling to measure Aß clearance from the synapse in AD. This will contribute to the growing body of literature that explores *APOE* genotype and Aß clearance mechanisms as potential therapeutic targets to prevent and treat AD.

Table 3. Study Samples

Control Cases

CASE #	AGE	SEX	AREA	*APOE* genotype
726	97	Female	A7	3/3
A09-109	40	Female	A7	3/3
39-13	78	Female	A7	3/3
2-11	97	Male	A7	3/3
9-12	95	Female	A7	3/3
7-09	63	Female	A7	3/3
15-09	82	Male	A7	3/3
773	90	Female	A7	3/3
779	89	Female	A40	3/3
789	105	Female	A7	3/3
1-13	99	Male	A7	3/4
12-09	85	Male	A7	3/4
810	81	Male	A7	4/4

Early AD (Braak I-III)

CASE #	AGE	SEX	AREA	*APOE* genotype
830	89	Female	A7	3/3
787	87	Female	A7	3/3
752	98	Male	A7	3/3
782	89	Female	A7	3/3
3-12	81	Male	A7	3/3
737	76	Female	A7	3/4
825	68	Female	A7	3/4
9-11	96	Female	A7	3/4
A05-133	92	Female	A7	3/4
819	80	Female	A7	3/4
835	78	Female	A7	3/4

Late AD (Braak V-VI)

CASE #	AGE	SEX	AREA	*APOE* genotype
21-10	97	Female	A7	3/3
31-11	98	Female	A7	3/3
5-13	99	Female	A7	3/3
18-10	92	Male	A7	3/3
39-10	90	Male	A7	3/3
16-11	75	Female	A7	3/3

21-11	96	Male	A7	3/3
37-11	94	Female	A7	3/3
33-12	56	Male	A7	3/3
36-12	56	Female	A7	3/3
808	99	Female	A7	3/4
731	83	Male	A7	3/4
809	79	Male	A7	3/4
849	87	Male	A7	3/4
A05-102	99	Female	A7	3/4
0909	80	Female	A7	3/4
33-09	82	Female	A7	3/4
776	93	Male	A39	3/4
23-10	85	Male	A7	3/4
38-13	74	Male	A7	3/4
37-13	71	Male	A7	4/4
720	63	Male	A7	4/4
2-12	64	Female	A39	4/4
28-12	76	Male	A39	4/4
811	59	Male	A7	4/4
4-10	79	Male	A7	4/4
11-13	78	Male	A40	4/4
805	65	Male	A7	4/4

Table 1: Case information for synaptosome samples.

A cohort of 52 human postmortem human brain samples with normal (n=13), early AD (n=11), late AD (n=28) from the pariertal cortex (A7, A39, A40) were used in the experiments detailed in this work. There are 28 female cases and 24 male cases with an average age of 88.

Figure 2.1

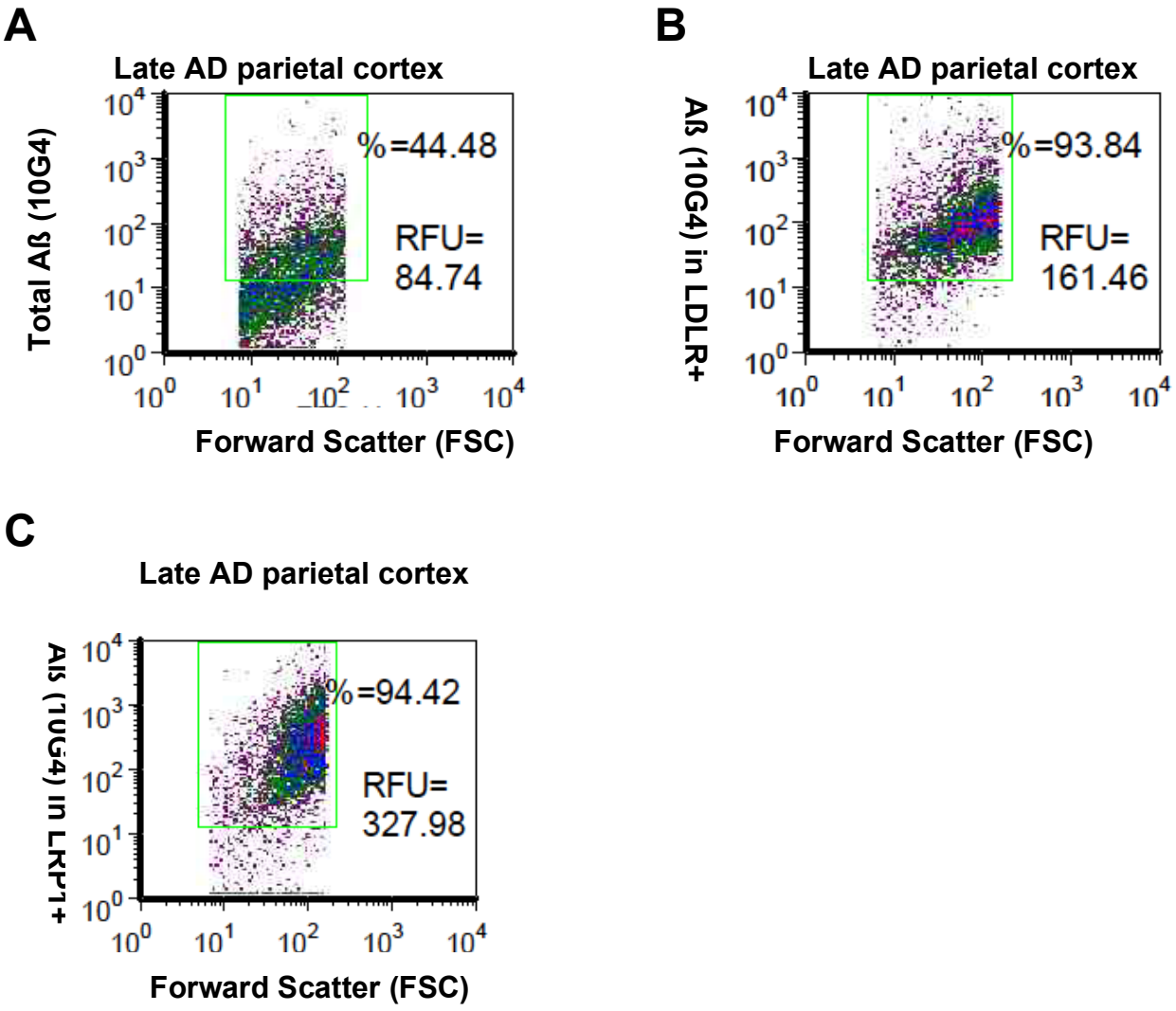


Figure 2.1: Aß is increased in LDLR- and LRP1-positive synapses compared to total Aß in late AD synaptosomes

Figure 2.2

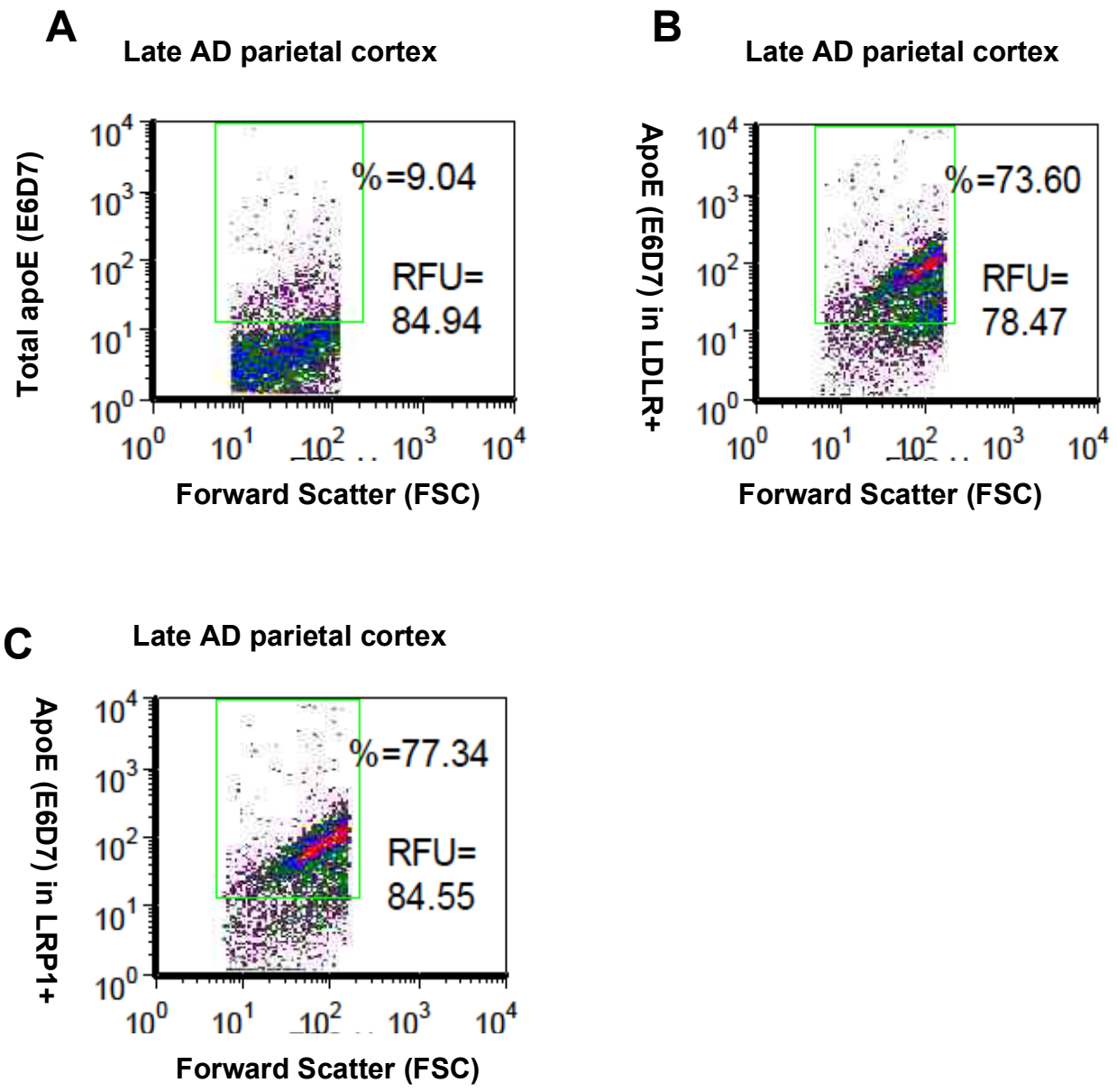


Figure 2.2: ApoE is increased in LDLR- and LRP1-positive synapses compared to total apoE in late AD synaptosomes

Figure 2.3

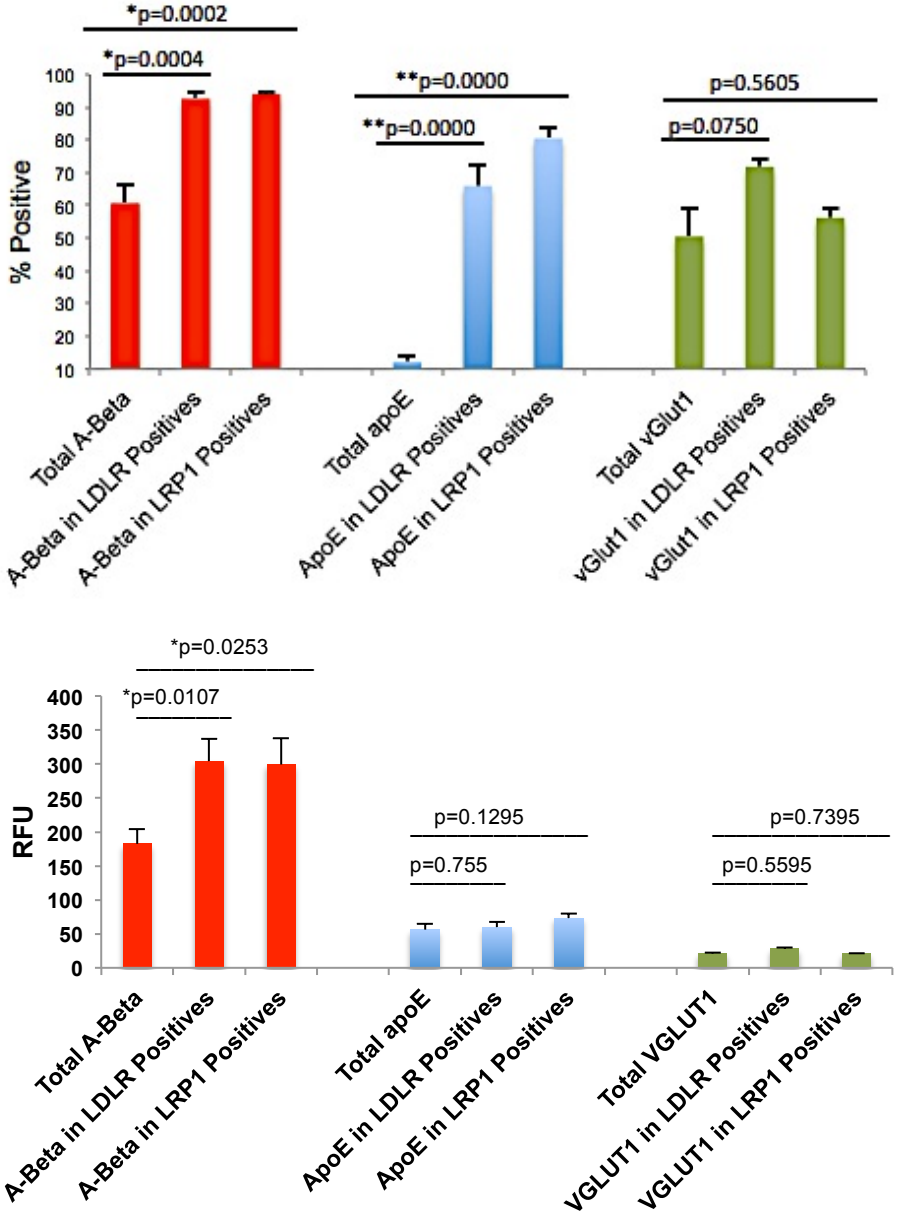
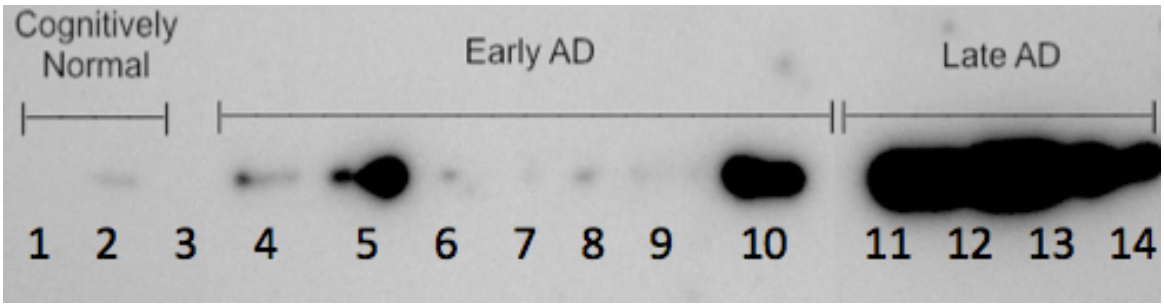


Figure 2.3: Aß and apoE are increased in LDLR- and LRP1-positive synapses.
Flow cytometry analysis demonstrates that Aß and apoE are enriched in LDLR and LRP1 synaptosomes in late AD (n=6). VGLUT1 was used as a negative control and demonstrated no significant differences in % positives in total VGLUT1 levels and VGLUT1 levels in LDLR- and LRP1-positive synapses. Data was collected from 10,000 terminals for each sample.

Figure 3.

A



B

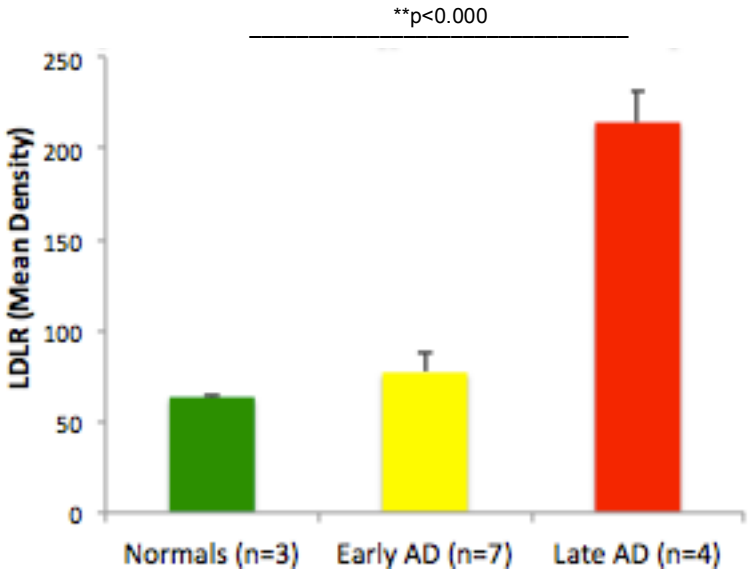
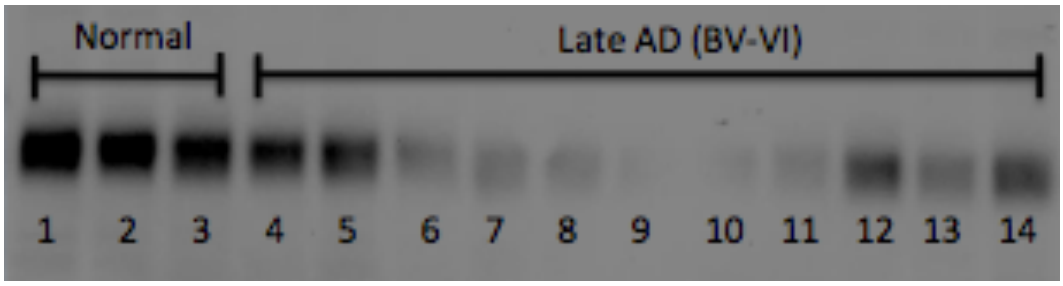


Figure 3: LDLR is increased in late AD versus normal synapses.
Western blot analysis demonstrates that LDLR mean density is significantly increased in late AD (n=4) versus normal synapses (n=3) and early AD synapses (n=7). All late AD samples contained the apoE e4 allele and thus, saw significant increases in late AD consistent with the literature.

Figure 4.1

A



B

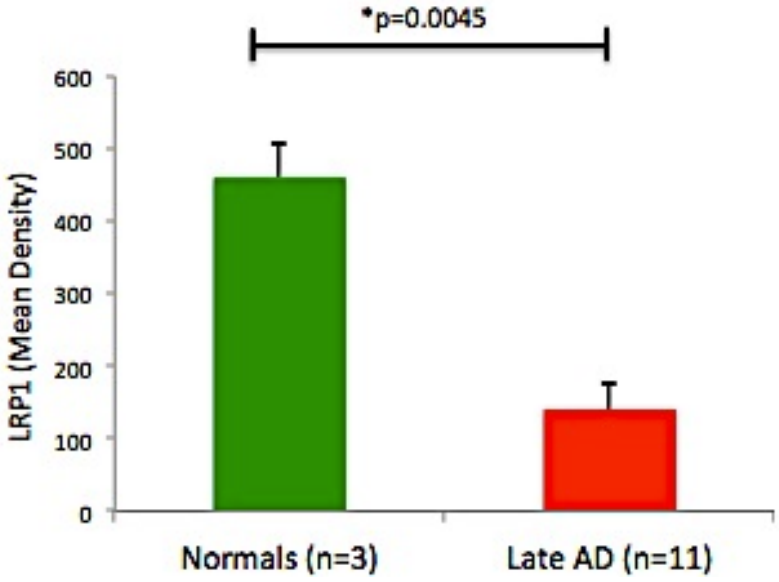


Figure 4: LRP1 is reduced in late AD versus normal synapses.
Western blot analysis demonstrates that LRP1 mean density is significantly increased in late AD (n=11) versus normal synapses (n=3) (p=0.0045).

Figure 4.2

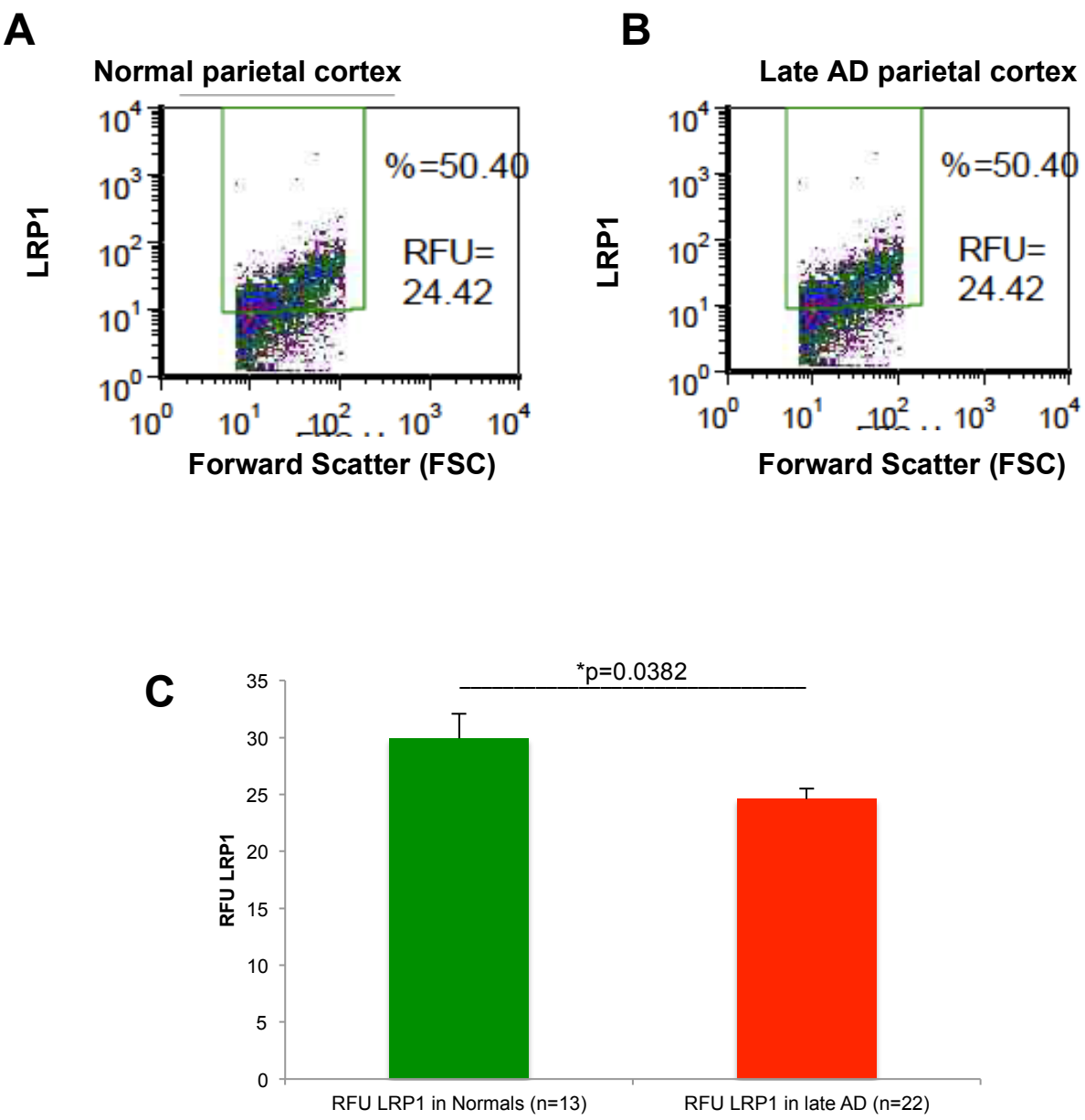


Figure 4.2: LRP1 is reduced in late AD versus normal synapses.
Flow cytometry analysis demonstrates that LRP1 RFU is significantly increased in late AD (n=22) versus normal synapses (n=13) (p=2). This is consistent with the literature and implies that LRP1 plays an important role in clearing Aß from the synapse in normal controls. Data was collected from 10,000 terminals for each sample.

Figure 5.1

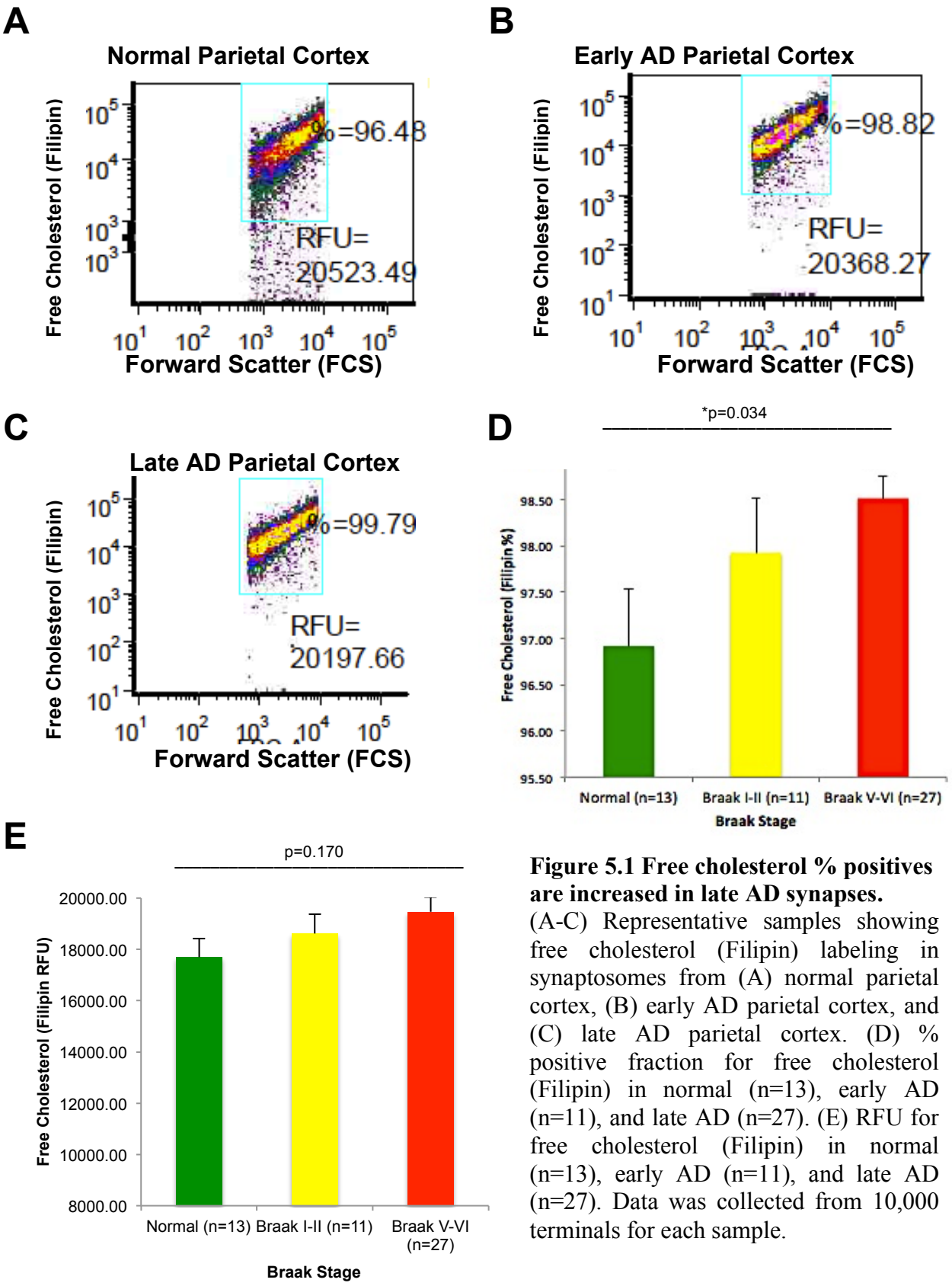


Figure 5.1 Free cholesterol % positives are increased in late AD synapses. (A-C) Representative samples showing free cholesterol (Filipin) labeling in synaptosomes from (A) normal parietal cortex, (B) early AD parietal cortex, and (C) late AD parietal cortex. (D) % positive fraction for free cholesterol (Filipin) in normal (n=13), early AD (n=11), and late AD (n=27). (E) RFU for free cholesterol (Filipin) in normal (n=13), early AD (n=11), and late AD (n=27). Data was collected from 10,000 terminals for each sample.

Figure 5.2

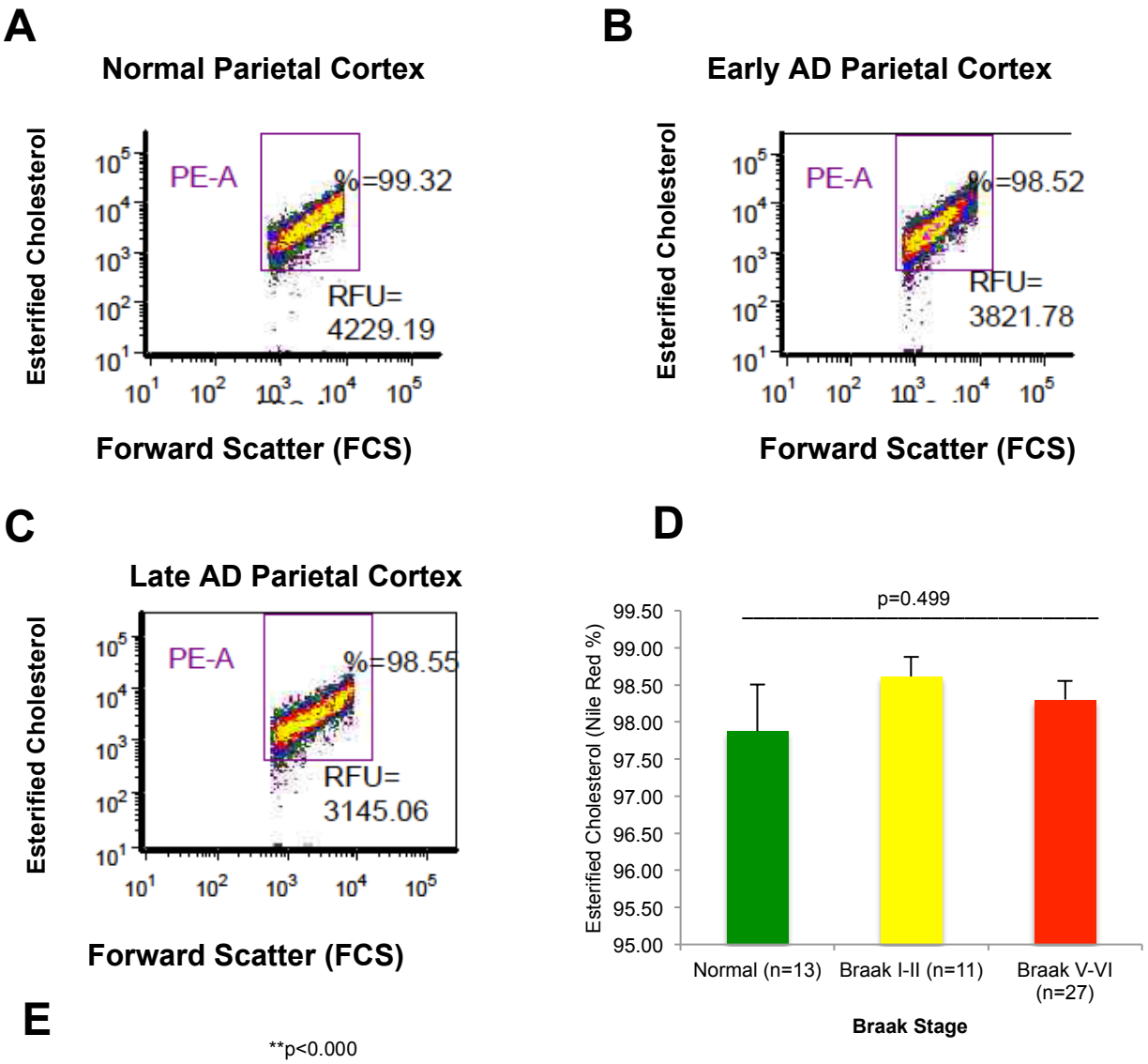


Figure 5.2 Esterified cholesterol RFU is reduced in late AD synapses.
(A-C) Representative samples showing esterified cholesterol (Nile Red) labeling in synaptosomes from (A) normal parietal cortex, (B) early AD parietal cortex, and (C) late AD parietal cortex. (D) % positive fraction for free cholesterol (Filipin) in normal (n=13), early AD (n=11), and late AD (n=27). (E) RFU of positive fraction for esterified cholesterol (Nile Red) in normal (n=13), early AD (n=11), and late AD (n=27). Data was collected from 10,000 terminals for each sample.

Figure 6.1

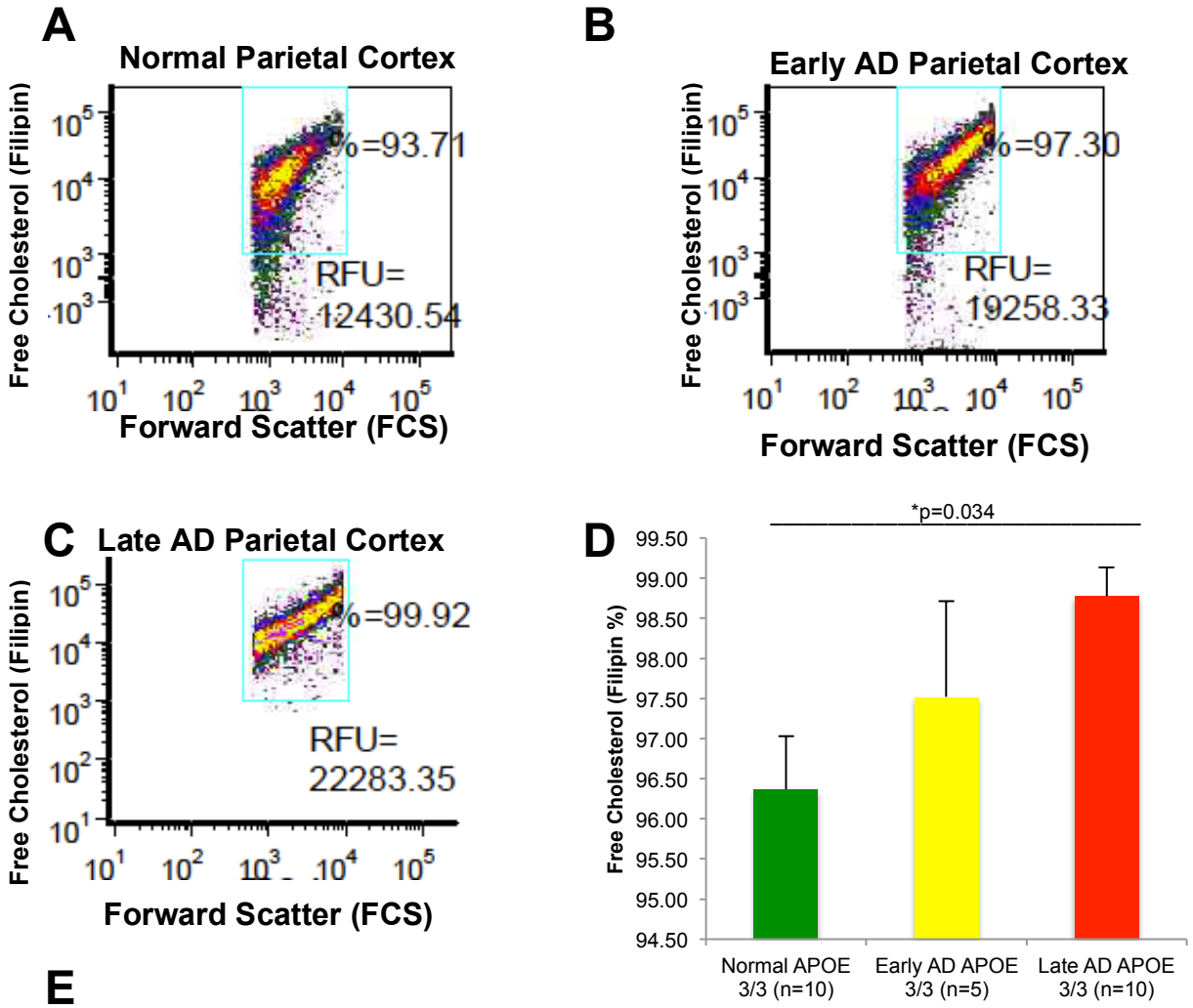


Figure 6.1 Free cholesterol is increased across Braak Stage among *APOE 3/3* synaptosomes.
(A-C) Representative samples showing free cholesterol (Filipin) labeling in synaptosomes from (A) normal parietal cortex, (B) early AD parietal cortex, and (C) late AD parietal cortex. (D) % of positive fraction for free cholesterol (Filipin) in normal (n=10), early AD (n=5), and late AD (n=10). (E) RFU of positive fraction for free cholesterol (Filipin) in normal (n=10), early AD (n=5), and late AD (n=10). Data was collected from 10,000 terminals for each sample.

Figure 6.2

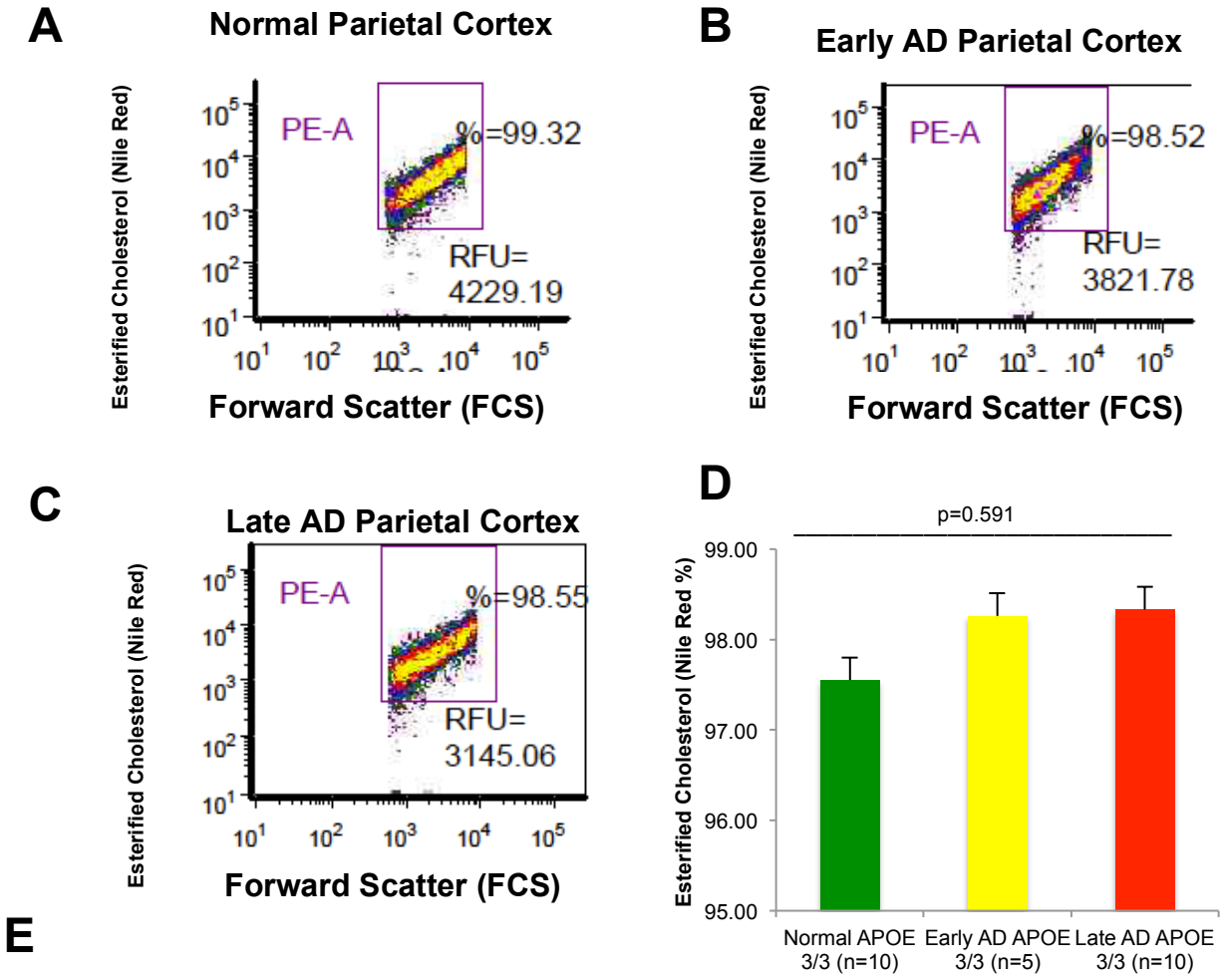


Figure 6.2 Esterified cholesterol RFU is reduced across Braak Stage among *APOE 3/3* synaptosomes.
(A-C) Representative samples showing esterified cholesterol (Nile Red) labeling in *APOE 3/3* synaptosomes from (A) normal parietal cortex, (B) early AD parietal cortex, and (C) late AD parietal cortex. (D) % positive fraction for esterified cholesterol (Nile Red) in normal (n=10), early AD (n=5), and late AD (n=10). (E) RFU of positive fraction for esterified cholesterol (Nile Red) in normal (n=10), early AD (n=5), and late AD (n=10). Data was collected from 10,000 terminals for each sample.

Figure 7.1

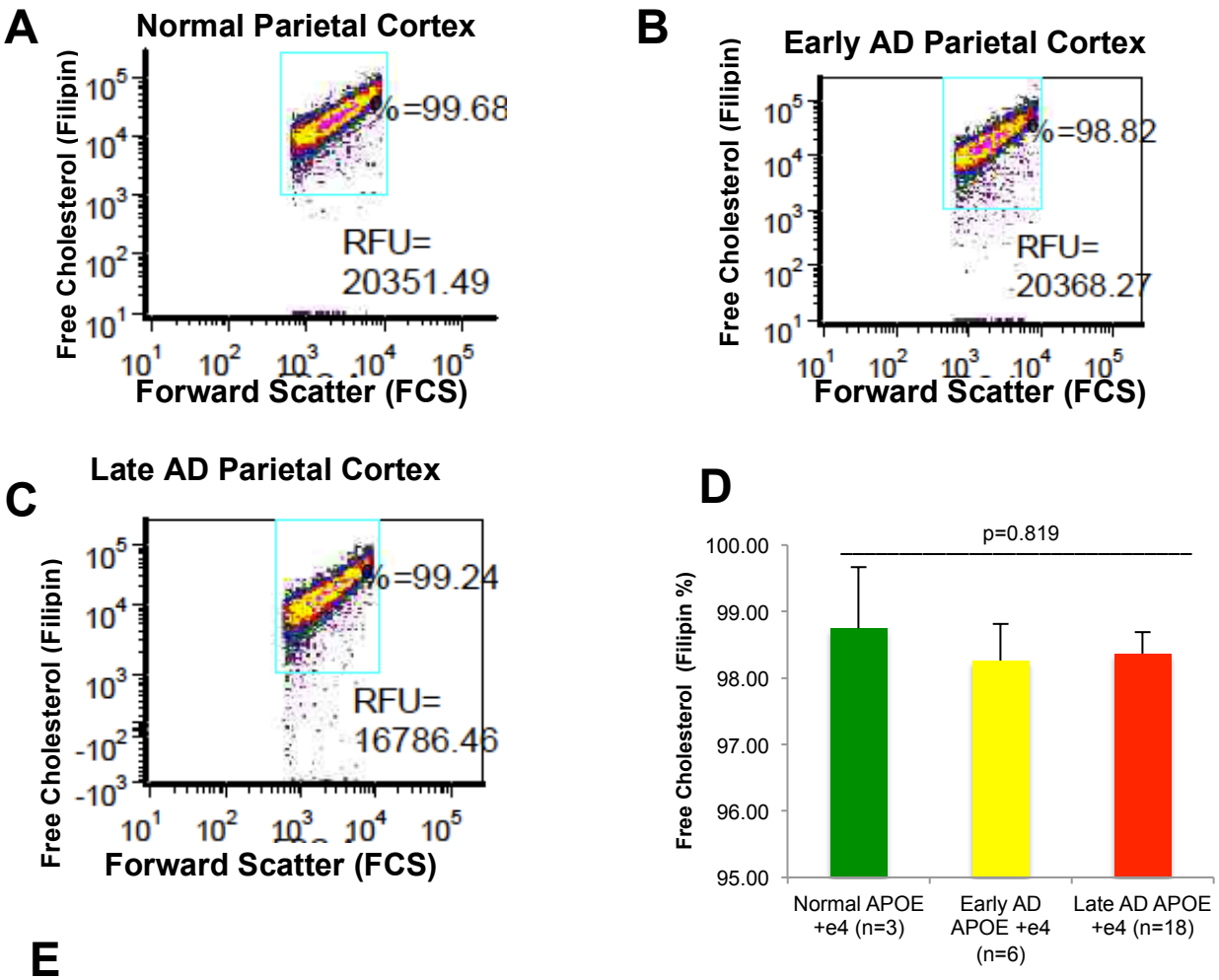


Figure 7.1 Free cholesterol level is not significantly altered across Braak Stage among *APOE +e4* synaptosomes. (A-C) Representative samples showing free cholesterol (Filipin) labeling in *APOE +e4* synaptosomes from (A) normal parietal cortex, (B) early AD parietal cortex, and (C) late AD parietal cortex. (D) % of positive fraction for free cholesterol in normal (n=3), early AD (n=6), and late AD (n=18). (E) RFU of positive fraction for free cholesterol in normal (n=3), early AD (n=6), and late AD (n=18). Data was collected from 10,000 terminals for each sample.

Figure 7.2

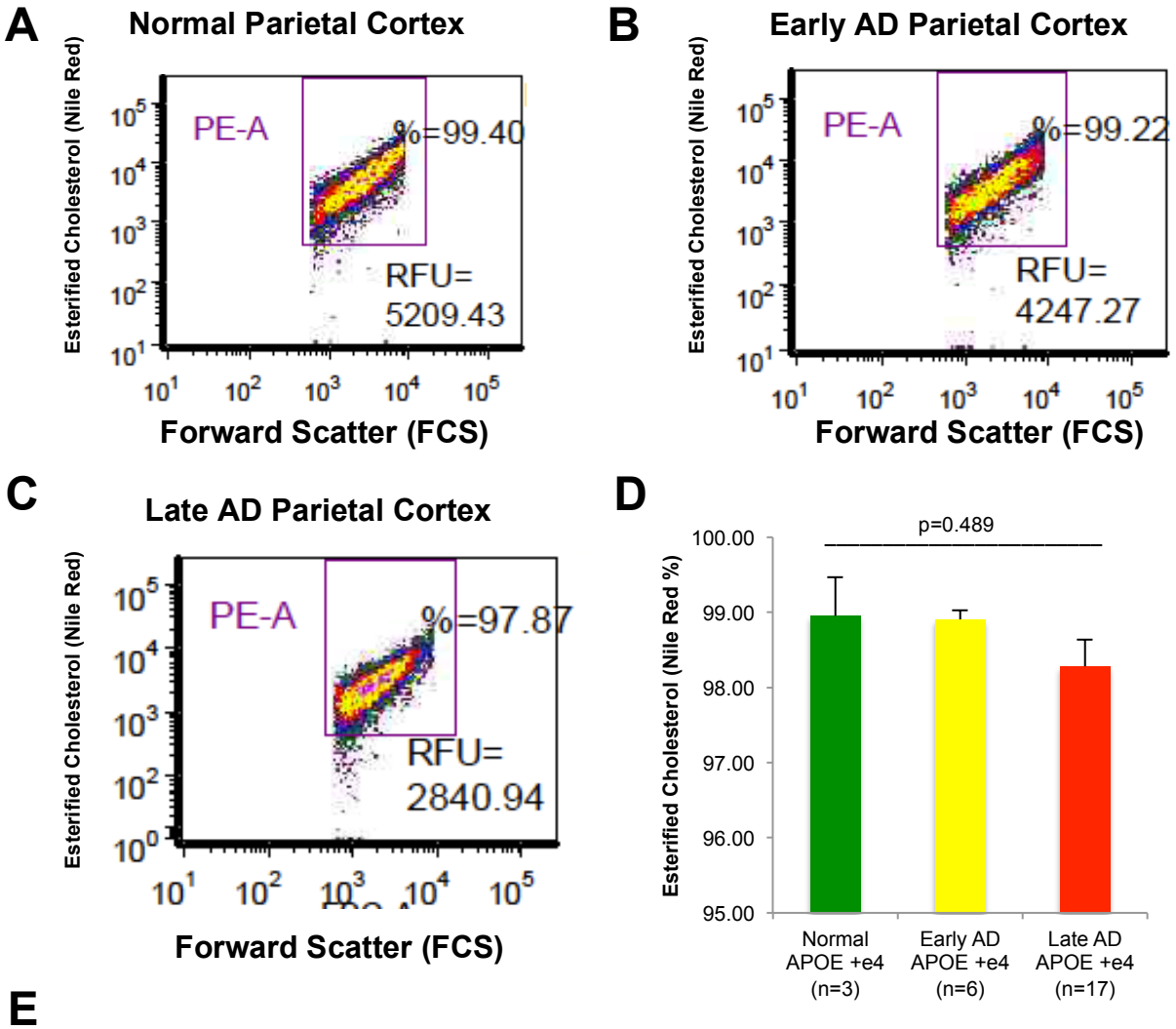


Figure 7.2 Esterified cholesterol level is not significantly altered across Braak Stage among *APOE +e4* synaptosomes. (A-C) Representative samples showing esterified cholesterol (Nile Red) labeling in *APOE +e4* synaptosomes from (A) normal parietal cortex, (B) early AD parietal cortex, and (C) late AD parietal cortex. (D) % of positive fraction for esterified cholesterol in normal (n=3), early AD (n=6), and late AD (n=17). (E) RFU of positive fraction for esterified cholesterol in normal (n=3), early AD (n=6), and late AD (n=17). Data was collected from 10,000 terminals for each sample.

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