

**REGULATION OF APOLIPOPROTEIN E SECRETION, LIPIDATION AND
RECYCLING IN THE CENTRAL NERVOUS SYSTEM**

by

Jianjia Fan

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Abstract

Lipid transport in the brain is coordinated by glia-derived lipoproteins that contain apolipoprotein E (apoE) as their primary protein. ApoE plays an important role in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD). ATP-binding cassette transporter A1 (ABCA1) effluxes cholesterol and phospholipids to apolipoprotein acceptors including apoE. ABCA1 is a key regulator of apoE levels and lipidation in the brain and deficiency of ABCA1 increases amyloid burden in AD mouse models. Translating these findings to potential therapies for AD will require a more thorough understanding of the biochemical nature of nascent apoE particles generated from glia and of lipoprotein remodeling in the CNS in general. In this thesis, I show that apoE is secreted from wild-type primary murine mixed glia as nascent lipoprotein subspecies ranging from 7.5 to 17 nm in diameter. Glia lacking ABCA1 secrete only one species of small particles (~8.1 nm), which are poorly lipidated, but can accept lipids to form the full repertoire of wild-type apoE particles. Inhibition of apoE receptor function blocks appearance of the 8.1 nm species, suggesting that this particle may arise through apoE recycling. Selective deletion of the LDL receptor significantly reduces the level of the 8.1 nm particles, suggesting that apoE is preferentially recycled through LDLR. These results suggest that nascent glial apoE lipoproteins are secreted through multiple pathways.

Modulating the expression, secretion or function of apoE may provide potential therapeutic approaches to protect the brain from chronic and acute damage. This thesis shows that progesterone and a synthetic progestin, lynestrenol, significantly induce apoE secretion from human CCF-STTG1 astrocytoma cells, whereas estrogens have negligible effects. Intriguingly, lynestrenol also increases expression of ABCA1 in human astrocytoma cells, primary murine glia, and immortalized murine astrocytes that express human apoE3. The progesterone receptor (PR) inhibitor RU486 attenuates the effect of progestins on apoE expression in astrocytoma but has no effect on ABCA1 expression in all glial cell models tested, suggesting that PR may participate in apoE but does not affect ABCA1 regulation. These results suggest that selective reproductive steroid hormones have the potential to influence glial lipid homeostasis through LXR-dependent and PR-dependent pathways.

Preface

For Chapter 2, a version of this chapter has been published. Fan J., Stukas S., Wong C., Chan J., May S., DeValle N., Hirsch-Reinshagen V., Wilkinson A., Oda MN., and Wellington CL. (2011) An ABCA1-independent pathway for recycling a poorly lipidated 8.1 nm apolipoprotein E particle from glia. *J Lipid Res.* 52(9):1605-16. All the work from this chapter was done in the Wellington Laboratory at UBC. The experiments were conceived and designed by Wellington CL and Fan J. The experiments and analyses were mostly performed by Fan J., with great technical support especially from Chan J. who conducted the bacterial production of RAP peptide and protein purification. Other co-authors also provided various technical supports. Fan J. produced all the figures and wrote the methods and results sections of the manuscript, whereas the introduction and discussion sections were originally drafted by Wellington CL.

For Chapter 3, a version of this chapter has been published. Fan J., Shimizu Y., Chan J., Wilkinson A., Ito A., Tontonoz P., Dullaghan E., Galea LA., Pfeifer T., and Wellington CL. (2013) Hormonal modulators of glial ABCA1 and apoE levels. *J Lipid Res.* 2013 Sep 2. [Epub ahead of print] (PMID: 23999864). The work from this chapter was done collaboratively between the Wellington Lab and the Centre for Drug Research and Development (CDRD), both located at UBC. The primary high-throughput screen for compounds that increase apoE secretion from human CCF-STTG1 astrocytoma cells was conducted at CDRD. Based on the screen results, Figure 3.1 was generated by Shimizu Y. from CDRD. The rest of the experiments in this study were designed and performed by Fan J., except for the quantitative RT-PCR analyses that were mostly done by our technician Chan J. The manuscript was written by Fan J. and edited by Wellington CL.

Since the work in this thesis involves using primary cell cultures produced from animal tissues, all the experiments were approved by the UBC Animal Care Committee (Animal Care # A09-0916, # A09-0288, # A13-0036) and the UBC Biosafety Committee (Biosafety # B09-0119). I have successfully completed the training requirements of the Canadian Council on Animal Care (CCAC) and National Institutional Animal User Training Program (NIAUT). My CCAC certificate number is 2027.

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List of Abbreviations

ABC	ATP-binding cassette transporter
AD	Alzheimer's disease
AICD	APP intracellular domain
Apo	apolipoprotein
ApoE	apolipoprotein E
ApoER2	apoE receptor-2
APP	amyloid precursor protein
A β	amyloid β
BACE1	beta-site APP-cleaving enzyme 1
BBB	blood-brain barrier
BSA	bovine serum albumin
CE	cholesteryl ester
CEE	conjugated equine estrogens
CETP	cholesteryl ester transfer protein
CM	chylomicron
CNS	central nervous system
CSF	cerebrospinal fluid
CTF	C-terminal fragment
DMSO	dimethyl sulfoxide
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
ERK	extracellular signal regulated kinase
FACS	fluorescent Activated Cell Sorting
FAD	familial early-onset Alzheimer's disease
FAS	fatty acid synthase
FBS	fetal bovine serum
FDG	fluorodeoxyglucose
GCM	glial conditioned media
HDL	high density lipoprotein
HMG CoA	hydroxymethylglutaryl CoA
HTS	high throughput screen
IDE	insulin-degrading enzyme
IDOL	inducible degrader of the LDLR
ISF	interstitial fluid
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoprotein
LDLR	LDL receptor
LOAD	late-onset Alzheimer's disease
LPS	lipopolysaccharide
LRP1	the LDL-receptor-related protein
LTP	long-term potentiation

LXR	liver-X-receptor
LXRE	LXR response element
MAPK	mitogen-activated protein kinase
MCI	mild cognitive impairment
MEF	mouse embryonic fibroblasts
MPA	medroxyprogesterone acetate
MRI	magnetic resonance imaging
NCoR	nuclear receptor co-repressor 1
NF- κ B	nuclear factor κ B
NFT	neurofibrillary tangle
OC	oral contraceptives
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PET	positron emission tomography
PL	phospholipid
PLTP	phospholipid transfer protein
PR	progesterone receptor
PS1	Presenilin 1
RAP	receptor-associated protein
RCT	reverse cholesterol transport
RIPA	radioimmunoprecipitation assay
RXR	retinoid X receptor
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SREBP1	sterol regulatory element-binding protein 1
SUMO	small ubiquitin-like modifier
TBI	traumatic brain injury
TC	total cholesterol
TD	Tangier Disease
VLDL	very low density lipoprotein
VLDLR	VLDL receptor
WT	wildtype

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To my parents.

To Sora and Roku.

Chapter 1: Introduction

1.1 Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disease that is the leading cause of senile dementia in developed countries. In 2010, more than 35.6 million people globally were suffering from AD. The World Alzheimer Report estimates that the global prevalence of AD will double every 20 years, reaching 115.4 million people by 2050 (Wimo & Prince, 2010). In Canada, AD makes up 63% of senile dementia and more than half a million Canadians were living with AD in 2010 (Dudgeon, 2010). In North America, 40% of seniors over 85 years of age are affected by this devastating disease. AD patients develop severe memory impairment. As the disease continues to progress, other cognitive and executive functions such as speaking, understanding and decision-making are gradually lost, thus greatly hindering the patient's ability to carry out daily activities and leaving them relying heavily on their caregivers (D. L. Price et al., 1998).

Despite the tremendous effort in studying the disease and searching for the cure, no effective treatment for AD has yet been successfully implemented. In North America, AD leads to caregiving costs of approximately \$50,000-\$85,000/year/person and incalculable emotional costs to patients and their caregivers (Alzheimer's Society of Canada). As the proportion of aged individuals in North America continues to increase, the burden of AD will become unsustainably high if effective disease-modifying therapies are not discovered and implemented.

1.1.1 Definition

Alzheimer's disease was first described in 1906 by Alois Alzheimer, a German physician who reported and described the clinical symptoms and neuropathological characteristics of the disorder based on the subsequent autopsy observation of one of his patients Auguste D (Alzheimer et al., 1995). The clinical symptoms Alzheimer described included severe mental impairment including reduced memory and comprehension, aphasia, disorientation, paranoia, hallucination and pronounced psychosocial impairment (Maurer et al., 1997). Pathologically, post-mortem examination on the patient's brain revealed evenly distributed brain atrophy resulted from the degeneration of neurons (Wenk, 2003). Later studies showed that the atrophy in AD brain is most pronounced in the frontal and temporal lobes. The hippocampus, the brain region that is critical for learning and memory, is located within the temporal lobe and is believed to be one of the earliest brain regions affected by AD (Braak & Braak, 1998). At a histological level, Alzheimer described extensive neuronal loss and two other distinct pathological features that define the AD brain: (a) the presence of abnormally prominent and thick neurofibrils ('tangles'), and (b) the presence of minute miliary foci of a 'peculiar substance' throughout the cortex ('plaques') (Alzheimer et al., 1995; Graeber & Mehraein, 1999). The substance found in the cortical plaques was later identified as *amyloid*. Today, the clinical and neuropathological observations initially made by Alzheimer have become the cornerstone for the diagnosis of AD.

Criteria for the clinical diagnosis of AD were established by the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA) working group in 1984. In the year 2011, the

criteria were updated by the Alzheimer's Association and the National Institute on Aging (NIA) based on the important advances in our understanding of AD in the past 27 years (Jack et al., 2011). According to the older criteria, a definitive diagnosis of AD must meet both clinical criteria for AD and post-mortem histological confirmation of the two neuropathological hallmarks, i.e. the extracellular amyloid plaques that consist mainly of aggregated amyloid β ($A\beta$) peptides and the intracellular neurofibrillary tangles (NFTs) consisting of hyperphosphorylated tau protein (McKhann et al., 1984; Morris, 1997). The revised 2011 criteria, however, made a semantic and conceptual distinction between pathophysiological process of AD (AD-P) and the various clinically observable syndromes (AD-C), since observations over the past 27 years indicate the clinical-pathological correspondence is not always consistent. For example, extensive AD pathology such as diffuse amyloid plaques can be present in the absence of any obvious clinical symptoms (Davis et al., 1999; Knopman et al., 2003; J. L. Price & Morris, 1999). The new guideline also formalized three stages of AD: (1) dementia due to Alzheimer's, (2) mild cognitive impairment (MCI) due to Alzheimer's, and (3) preclinical (presymptomatic) Alzheimer's as a newly recognized stage of the disease, characterized by distinct changes in AD relevant biomarkers. Identifying the preclinical stage of AD is particularly gaining attention since if researchers ever succeed in developing treatments that can slow or stop the progression of AD, such treatment may be most effective if implemented as early as possible.

A major change within the new AD criteria is the incorporation of AD biomarkers in living patients. Biomarkers are physiological, biochemical or anatomic parameters that can be measured in living patients, which reflect specific features of the AD-P processes. Today, the

term biomarker refers to both fluid analytes and imaging measures. Because elevations in brain levels of A β are more specific to AD than alterations in tau, the revised AD guidelines divide the five most established AD biomarkers into two major categories: (1) *biomarkers of A β accumulation*, including low CSF A β 42 levels and abnormal tracer retention on amyloid PET imaging; and (2) *biomarkers of neuronal degeneration or injury*, including elevated CSF tau, decreased fluorodeoxyglucose (FDG) uptake in temporoparietal cortex on PET scan, and atrophy with a specific topographic pattern on structural magnetic resonance imaging (MRI) (Jack et al., 2011). Because the onset and progression of AD biomarkers likely follow an ordered temporal pattern, the role of these biomarkers differs in each of the three stages of AD. For example, in the preclinical stage, biomarkers are used to establish the presence of AD-P in research subjects with no or very subtle clinical symptoms, whereas in the MCI and dementia phase, clinical diagnoses are primary and biomarkers are complementary. Recent evidence suggests that alteration of A β biomarkers may occur as early as 10 to 20 years before noticeable clinical symptoms, whereas biomarkers of neurodegeneration become dynamic at a later point and shortly before clinical symptoms first manifest (Jack et al., 2010a; Jack et al., 2009; Mormino et al., 2009; Perrin et al., 2009).

1.1.2 Neuropathology of AD

Though several changes based on new research findings of AD have been made to the 2011 revised AD criteria as mentioned above, the fundamental characteristics of AD pathology, i.e. the presence of amyloid plaques in cortex and intracellular neurofibrillary tangles, continue to define the neuropathological entity of AD (Jack et al., 2011).

Neurofibrillary tangles occur in selected neuronal cell bodies and can be detected by silver stain (Kumar et al., 2005; Selkoe & Podlisny, 2002). NFTs are formed by hyperphosphorylation of a microtubule-associated protein known as tau (Grundke-Iqbal et al., 1986; Iqbal et al., 1989; Kosik et al., 1986). Tau is a phosphoprotein that exists in a non-phosphorylated form as well as a phosphorylated state (Lindwall & Cole, 1984b). Phosphorylation of tau has been shown to impair its ability to bind to and promote microtubule polymerization (Cho & Johnson, 2004; Lindwall & Cole, 1984a; Schneider et al., 1999). Microtubules are one of the three main kinds of cytoskeletal filaments and play key roles in intracellular transport and trafficking. Tau is mostly found in the axons of neurons where it stabilizes microtubules to promote efficient movement of cargo over long distances (Binder et al., 1985). About 45 different phosphorylation sites on tau have been identified (Ksiezak-Reding et al., 1992). During the progress of AD, tau becomes hyperphosphorylated, which leads to detachment from microtubules and an elevated concentration of soluble free tau. Free tau can undergo self-association and aggregation to form paired-helical filaments that ultimately become the neurofibrillary tangles. This is known as the *tau hypothesis of AD*. However, the precise mechanism of tau hyperphosphorylation and NFT formation is not completely understood.

The presence of NFTs (tauopathy) is not a unique pathology to AD. NFTs can also be found in other dementias or neurodegenerative conditions such as frontotemporal dementia with parkinsonism on chromosome 17 (FTDP-17) (Selkoe & Podlisny, 2002), chronic traumatic encephalopathy (CTE) (Roberts, 1988), Lytico-Bodig disease (Hof et al., 1994) and tangle-predominant dementia (Jellinger & Attems, 2007; Santa-Maria et al., 2012). On the other hand, amyloid plaques are thought to be more specific to AD (Glenner & Wong, 1984). Amyloid

deposits are composed of A β peptides. Amyloid plaques that are deposited in the neural parenchyma can be classified into *diffuse plaques* and *neuritic plaques*. Diffuse plaques are composed of non-fibrillar A β deposits and do not exhibit dystrophic neurites whereas neuritic plaques are compacted fibrillar amyloid core surrounded by dystrophic neurites. In approximately 80% of AD patients, amyloid plaques are also found in cerebral blood vessels (D. L. Price et al., 1998). A β peptides are generated from sequential proteolytic cleavage of the *amyloid precursor protein* (APP) (Selkoe, 2001). APP is a type I transmembrane glycoprotein that are conserved across a number of species. Human APP exists as one of three different isoforms containing 695, 751 or 770 amino acid residues (Buxbaum et al., 1990; Gandy et al., 1988; Kang et al., 1987; Kitaguchi et al., 1988; Ponte et al., 1988). APP695 is primarily expressed in the brain whereas the 751 and 770 amino acid isoforms are expressed in other tissues (Kang et al., 1987; Manning et al., 1988). The N-terminal of APP is localized extracellularly whereas its C-terminal faces the cytosol.

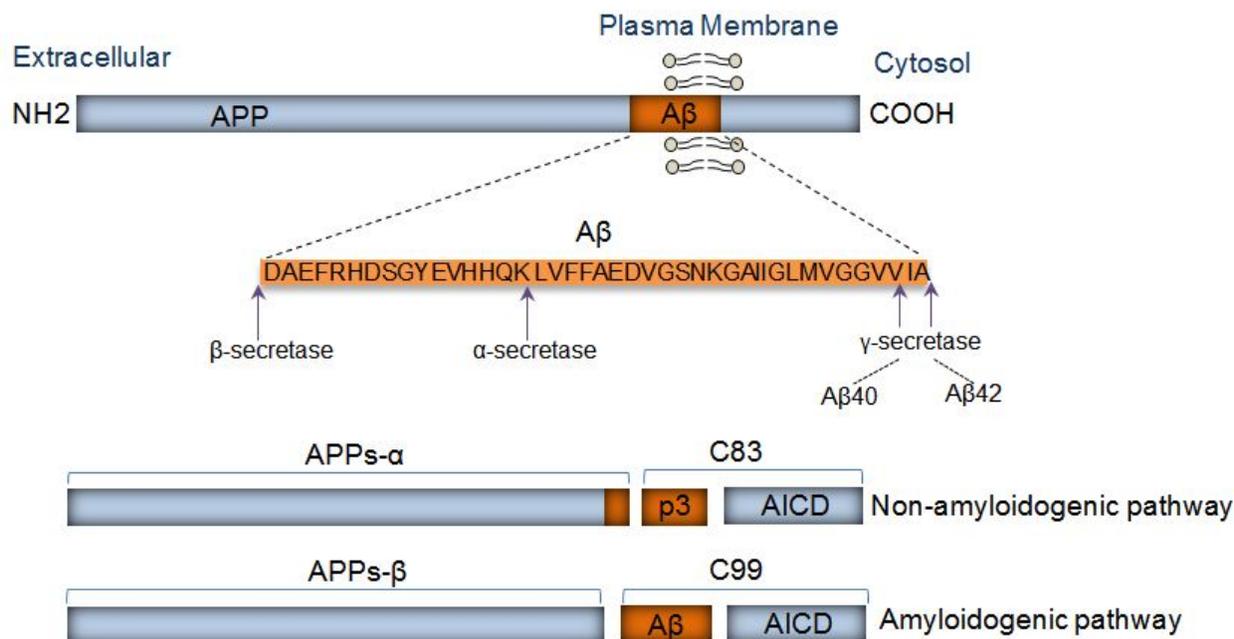


Figure 1.1: APP processing pathways.

APP is cleaved by three major secretases: α -secretase, β -secretase and γ -secretase. In the non-amyloidogenic pathway, cleavage of APP by α -secretase releases a large soluble ectodomain of APP (APPs- α) and an 83-aa C-terminal fragment (C83) in the membrane which is further cleaved by γ -secretase to release a p3 peptide and an APP intracellular domain (AICD). In the amyloidogenic pathway, APP is cleaved by β -secretase to generate an APPs- β domain and a C99 fragment. C99 is then processed by γ -secretase to produce A β peptides, the majority of which are 40- or 42-aa in length.

The APP holoprotein undergoes a series of enzymatic cleavages mediated by α -secretase, β -secretase and the γ -secretase complex (De Strooper et al., 1998; Evin et al., 1995; Lammich et al., 1999; Vassar et al., 1999). Mature APP is processed by either one of two cleavage pathways: a non-amyloidogenic pathway that precludes the formation of A β (Naslund et al., 1994) and an amyloidogenic pathway that produces A β (Estus et al., 1992; Golde et al., 1992) (**Fig. 1.1**). Most of the wild-type APP is processed by the non-amyloidogenic pathway, in which APP is first cleaved by α -secretase to release a large, soluble, secreted ectodomain (APPs- α) and a

membrane-bound C-terminal fragment (CTF) of 83 amino acid residues (C83). The C83 domain is then further cleaved by the γ -secretase complex within the transmembrane region to release the non-amyloidogenic peptide, p3 and the APP intracellular domain (AICD) (Naslund et al., 1994). In the alternative amyloidogenic pathway, APP is first cleaved extracellularly by β -secretase at different site than α -secretase, which gives rise to the soluble APPs- β domain and the membrane bound fragment C99. C99 is then again cleaved by the γ -secretase complex within the transmembrane region, producing the A β peptide and the AICD (Estus et al., 1992; Golde et al., 1992). The length of A β is reported to range from 37 to 43 amino acids, though A β 40 and A β 42 are quantitatively the most important. A β 42 is more hydrophobic and aggregates more easily when compared to A β 40. A β 42 is demonstrated to be required for the formation of amyloid (McGowan et al., 2005), as it constitutes most of the immature plaques found in AD brains. The immature plaques subsequently develop into neuritic plaques by recruiting A β 40 (Iwatsubo et al., 1994). α and β cleavages are two competing pathways that are mutually exclusive. Genetic mutations that result in familial early-onset AD (FAD) increase the proportion of APP being cleaved by the amyloidogenic β -pathway rather than the non-amyloidogenic α -pathway (Hardy & Selkoe, 2002).

AD patients also manifest cerebral atrophy and neurodegeneration in addition to plaques and tangles. Interestingly, entorhinal cortical neurons and CA1 hippocampal neurons appear to be the most vulnerable in AD brains (Gomez-Isla et al., 1997), although it is unclear why there is region-specific susceptibility in AD brains. Amongst all the neuropathological features in AD, synaptic/neuronal loss is the best correlate for memory dysfunction, yet the precise mechanisms that lead to the loss of synapses and neurons remain to be elucidated. It is also unclear why

amyloid pathology of AD and neurodegenerative pathology of AD (in the form of NFT formation and synaptic/neuronal loss) occur on different time scales rather than developing simultaneously (Ingelsson et al., 2004).

1.1.3 The Amyloid cascade hypothesis

The amyloid cascade hypothesis, or amyloid hypothesis for short, is a favored yet controversial hypothesis that has been put forward to account for the cause of AD (Hardy & Selkoe, 2002). This hypothesis states that accumulation of the insoluble aggregates of A β protein in the brain tissue is the primary causative factor that initiates the pathological cascade that ultimately results in all of the pathological hallmarks of AD (Hardy & Selkoe, 2002). The hypothesis was postulated based on the discoveries from trisomy 21 cases and AD-linked mutations in the APP gene (Goate et al., 1991; Lemere et al., 1996; Teller et al., 1996; Tokuda et al., 1997). Mutations in the APP, Presenilin-1 and Presenilin-2 (components of the γ -secretase complex) genes are found to cause FAD with increased generation of A β peptides (Citron et al., 1997; Scheuner et al., 1996; Thinakaran et al., 1996). Down's syndrome patients have an extra copy of chromosome 21 on which the human APP gene is located. DS patients inevitably develop AD neuropathology by age 40, further supporting the amyloid hypothesis. Moreover, the major genetic risk factor for late-onset AD (LOAD), APOE4, also leads to excess amyloid accumulation in AD brains (Polvikoski et al., 1995). In a variety of experimental paradigms, A β peptides are found to be neurotoxic (Selkoe, 2001).

Although the amyloid hypothesis provided a broad framework to explain AD pathogenesis over the past decade, there remain some controversies about the validity of this hypothesis. For

example, the most common concern of the amyloid hypothesis is that the number of amyloid plaques does not correlate well with the degree of cognitive impairment (Giannakopoulos et al., 2003) as there are reports in which subjects with substantial amyloid plaques do not present with any clinical symptoms of AD (Armstrong, 1994). More recent studies using biochemical assays demonstrate that A β loads correlate more closely with cognitive impairment than the histologically-detected amyloid plaques (Cairns et al., 2009; Jack et al., 2009; Jack et al., 2010b; Morris et al., 2009). Several lines of evidence have converged to suggest that A β oligomers, rather than A β monomers or insoluble A β fibrils in the form of neuritic plaques, that may cause neurotoxicity and synaptic dysfunctions in AD (Kamenetz et al., 2003; Wei et al., 2010). It is found that transgenic mice overexpressing human mutant APP genes develop cognitive impairment prior to detectable plaque formation (Games et al., 1995; Hsiao et al., 1996). Therefore, it is argued that the role of soluble oligomeric A β as the toxic agent in AD pathogenesis should be incorporated into a revised amyloid hypothesis. Nevertheless, the available genetic risk data overwhelmingly point to some aspect of A β amyloid pathway as the initiating or at least a very early pathophysiological event in the disease cascade (Hardy & Selkoe, 2002). While familial AD can be characterized by overproduction of A β , late-onset sporadic AD may be characterized by decreased clearance of A β (Mawuenyega et al., 2010).

1.1.4 AD and the cholesterol connection

The brain is the most cholesterol-rich organ in the body, which contains ~25% of total body cholesterol in only 2% of total body weight (Dietschy & Turley, 2001). Cholesterol is a major component of neuronal and glial membranes and of myelin that sheathes axons. Nearly all brain cholesterol is synthesized *in situ*, as essentially no cholesterol carried on peripheral lipoproteins

enters the brain across the blood-brain-barrier (BBB) (Dietschy & Turley, 2001). As brain cells cannot degrade cholesterol, excess brain cholesterol is converted to 24S-hydroxycholesterol which readily traverses the BBB and is delivered to the peripheral circulation for eventual excretion via the liver (Bjorkhem et al., 1997; Bjorkhem et al., 1998; Pitas et al., 1987a).

Several lines of evidence support the hypothesis that cholesterol homeostasis is involved in the pathogenesis of AD. First, apolipoprotein E (apoE), the major lipid carrier in brain, is the most established genetic risk factor for late-onset sporadic AD, and genetic variations in the human apoE gene account for more than 95% of AD cases. The role of apoE in AD pathogenesis will be discussed in detail in Section 1.3.4. In the sections below, the role of cellular cholesterol levels on APP processing, plasma lipid alteration in AD, and connection of statin use and AD prevalence will be discussed.

1.1.4.1 Cholesterol and APP processing

The proteolytic cleavage of APP occurs both within and very close to its transmembrane domain. Notably, all the APP secretases are also integral membrane bound proteins; especially the proteolytic activity of γ -secretase takes place within the hydrophobic membrane domain. The association of APP and BACE1 (a component of β -secretase) in cholesterol-rich lipid rafts is shown to affect the proteolytic activity of γ -secretase and β -cleavage of APP (Ehehalt et al., 2003). In vitro experiments using chemical or pharmacological methods to manipulate cholesterol have shown that cholesterol depletion reduces A β production (Bodovitz & Klein, 1996; Buxbaum et al., 2001; Ehehalt et al., 2003; Fassbender et al., 2001; Kojro et al., 2001; Simons et al., 1998), whereas high intracellular cholesterol levels increase the amyloidogenic

processing of APP (M. Burns et al., 2003; Refolo et al., 2000; Shie et al., 2002; Wahrle et al., 2002). These observations suggest that alteration in the neuronal lipid environment may have considerable consequences for APP processing. Interestingly, A β itself has been implicated in modulating cholesterol efflux, intracellular cholesterol synthesis, esterification and distribution (Grimm et al., 2005; Liu et al., 1998; Michikawa et al., 2001), suggesting possible feedback mechanisms between A β and cholesterol metabolism. However, further studies are needed to evaluate the possible role of A β on brain lipid homeostasis.

1.1.4.2 Plasma lipids and AD

In AD animal models, diet-induced hypercholesterolemia exacerbates amyloid deposition in brain (Refolo et al., 2001; Refolo et al., 2000; Shie et al., 2002; D. L. Sparks et al., 1994). Possible mechanisms underlying these effects could be the altered processing of APP due to increased cholesterol levels, or induction of a proinflammatory state in the cerebrovasculature (Shie et al., 2002; D. Larry Sparks et al., 2000). However, epidemiological human studies investigating the association of plasma cholesterol levels and AD risk are inconclusive, particularly for those that use total cholesterol levels as their primary outcome measure (Mason et al., 1992; Notkola et al., 1998; Pappolla et al., 2003; Roher et al., 1999; Whitmer et al., 2005). Some clinical studies suggest a positive correlation between increased plasma cholesterol in mid-life and the development of dementia, but this association is less clear for AD in particular (Kalmijn et al., 2000; Kivipelto et al., 2002; Notkola et al., 1998; Tan et al., 2003; Whitmer et al., 2005). Interestingly, A β is found on several peripheral lipoproteins in the circulation (Biere et al., 1996; Koudinov et al., 2001; Koudinov et al., 1998; Koudinov et al., 1994; Kuo et al., 1999)

and the level of lipoprotein-free A β is reported to be elevated in AD patients, suggesting that impaired lipoprotein-mediated clearance of A β could contribute to AD (Matsubara et al., 2004).

1.1.4.3 Cholesterol-lowering drugs and AD

Statins are inhibitors of hydroxymethylglutaryl CoA reductase (HMG CoA reductase), an enzyme that catalyses the rate-limiting step in sterol biosynthesis. Two retrospective epidemiological studies in 2000 showed a reduced AD prevalence and risk in patients taking statins (Jick et al., 2000; Wolozin et al., 2000). A 2007 retrospective study involving 4.5 million US subjects suggests that the brain-penetrant statin, simvastatin, may be particularly effective in lowering dementia incidence (Wolozin et al., 2007). Inhibition of cholesterol biosynthesis also lowers amyloid burden in some AD animals (Fassbender et al., 2001; Refolo et al., 2001) and decreases A β generation in vitro (Kojro et al., 2001; Simons et al., 1998). However, clinical trials evaluating the efficacy of statins to affect A β levels or AD incidence showed that there is no protective effect of statin use on cognitive decline (G. Li et al., 2004; Santanello et al., 1997; Shepherd et al., 2002; Zandi et al., 2005). A prophylactic or therapeutic role for statins remains controversial at this point. Further, long-term, highly powered prospective studies will be required to determine the effectiveness of statin treatment in the prevention and treatment of AD. Nevertheless, these findings have raised interest in understanding how cholesterol and lipid metabolism may influence the pathogenesis of AD.

1.1.5 ApoE: the most validated genetic risk factor of AD

ApoE is the main apolipoprotein found in the central nervous system (CNS) and has been reported to be co-localized with amyloid plaques in AD brains (Namba et al., 1991; Wisniewski

& Frangione, 1992). In 1993, apoE was discovered to be a genetic risk factor for AD and still remains the most important risk factor for late-onset AD today (Corder et al., 1993; Poirier et al., 1993; Strittmatter & Roses, 1995). In human, apoE exists in three major isoforms: apoE2, apoE3, and apoE4 (Weisgraber et al., 1981; Zannis & Breslow, 1980, 1981). Though the isoforms differ by only one or two amino acid residues at position 112 and 158, these differences profoundly change apoE structure and function (Mahley et al., 2006). How isoform differences may affect the pathogenesis of AD will be discussed in detail in later sections (Section 1.3). Meta-analysis of all published AD association studies up to 2010 (AlzGene, 2010) shows that apoE3 (cys112, arg158) is the predominant isoform in humans, present in 79% of the healthy population, whereas apoE2 (cys112, cys158) is the least prevalent isoform (7%). About 14% of the healthy population possess the allele (APOE ϵ 4) for apoE4 (arg112, arg158) (**Table 1.1**). Studies on the polymorphisms of APOE and AD quickly revealed that carriers of APOE ϵ 4 alleles are overrepresented in AD patients (Strittmatter et al., 1993a) (**Table 1.1**). People who carry at least one APOE ϵ 4 allele have an increased risk for AD by 2-3 fold compared to individuals with no APOE ϵ 4 allele. The risk is increased about 12-fold in carriers of two APOE ϵ 4 alleles (Poirier et al., 1993; Roses, 1996; Saunders et al., 1993). APOE ϵ 4 is also associated with an earlier age onset of AD (Gomez-Isla et al., 1996; Roses, 1996), as the average of age onset drops from 84-years of age in non APOE ϵ 4 carriers to 68-years of age in carriers of two copies of APOE ϵ 4 (Corder et al., 1993). On the other hand, the APOE ϵ 2 allele is associated with a lower risk of developing AD (Corder et al., 1994; Farrer et al., 1997).

Frequency	APOE Allele			ApoE Genotype					
	$\epsilon 2$	$\epsilon 3$	$\epsilon 4$	2/2	2/3	3/3	2/4	3/4	4/4
Control	0.07	0.79	0.14	0.007	0.11	0.623	0.019	0.222	0.019
AD	0.04	0.58	0.38	0.003	0.046	0.343	0.026	0.434	0.148

Table 1.1: Summary of apoE allele and genotype distribution in healthy and AD population

Although several roles of apoE in AD pathogenesis have been proposed (which will be discussed in Section 1.3.4), the exact mechanisms by which apoE influences the development of AD, particularly in an isoform-specific manner, remain to be fully understood. This thesis further explores the biochemical nature and regulation of apoE in CNS, which may provide insights for a better understanding of its function and implication in pathogenesis of neurodegenerative diseases and conditions.

1.2 Lipoprotein overview

The primary function of apoE is to serve as a protein component of lipoproteins. Lipoproteins are water soluble complexes of proteins (apolipoproteins) and lipids that transport hydrophobic lipids in the aqueous body fluids such as plasma, lymph, interstitial fluid (ISF) and cerebrospinal fluid (CSF). The major function of lipoproteins is to deliver lipids to cells or tissues that use or store lipids from those that synthesize them. In a typical structure of a lipoprotein particle, the apolipoprotein component surrounds, stabilizes and solubilizes their fatty cargo. Plasma lipoproteins are the most well studied pool of lipoproteins in the human body. On the other hand, lipoproteins found in lymph, ISF and CSF are less well characterized. The role of lipoproteins in CNS function has just started to be appreciated in the past two decades as apoE polymorphism was found to be an important risk factor associated with several neurodegenerative diseases and

brain injury conditions. One of the goals of this thesis work is to expand our knowledge on the generation and modification of apoE-containing CNS lipoproteins.

1.2.1 Peripheral lipoproteins

Peripheral lipoproteins are synthesized in the liver and the intestine. They can be assembled at the cell membranes from cellular lipids and exogenous apolipoproteins, or they can also arise from metabolic changes of precursor lipoproteins. Circulating lipoproteins are highly dynamic. They undergo enzymatic reactions of lipid transfers, exchange of soluble apolipoproteins, and conformational changes of the protein components in response to the lipid compositional changes. Eventually, plasma lipoproteins are taken up via receptor-mediated endocytosis or other mechanisms, and catabolized by liver, kidney or other peripheral tissues. The most common classification of plasma lipoproteins is based on their hydrated density.

Based on the relative contents of protein and lipid that determine the densities of the particles, lipoproteins can be classified into chylomicrons (CM), very low density (VLDL), low density (LDL) and high density (HDL) lipoproteins. The least dense CM have only about 1-2% protein whereas HDL have about 50% proteins by weight. The sizes (diameters) of lipoprotein particles are inversely correlated with their densities, hence CM are the largest lipoproteins with diameters ranging from 200 nm to 600 nm, while the smallest HDL particles are about 7 nm in size (**Fig. 1.2**). Although the proportion of lipid composition varies among lipoprotein classes, the general structural organization is similar for all the classes (**Fig. 1.2**). The apolipoproteins and amphipathic lipids, mostly phospholipids (PL) and unesterified cholesterol, form a 2 nm thick shell on the surface of the spherical particles, whereas the enclosed core consists of neutral

lipids, mainly triglycerides and cholesteryl esters (CE). Each lipoprotein class has its own characteristic apolipoprotein components. For example, the non-exchangeable apoB48 is the main apolipoprotein found on CM whereas apoB100 is the principal protein of VLDL and LDL. On the other hand, the exchangeable apolipoprotein apoA-I plays critical roles in HDL metabolism. ApoE is also an exchangeable apolipoprotein that can transfer between lipoprotein particles and serves as secondary protein component of CM, VLDL and HDL (**Fig. 1.2**).

The principal functions of the lipoprotein classes are determined by their apolipoprotein and lipid composition. The most lipid-rich particles CM are synthesized in the intestines for the transport of dietary triglycerides to various tissues. VLDLs are synthesized in the liver and transport endogenous triglycerides from liver to other tissues. LDLs arise from the metabolic transformation of VLDL in circulation and delivers CE to the liver and other peripheral tissues. Recipient tissues for lipid uptake include adipose tissue where lipids are stored and skeletal muscles where triglycerides are used as a source of energy. In contrast, the smallest, most protein-rich particles, HDL, participate primarily in the removal of excess cholesterol from cells and transport it to the liver and steroidogenic tissues for metabolism and excretion (D. E. Vance & Vance, 2008).

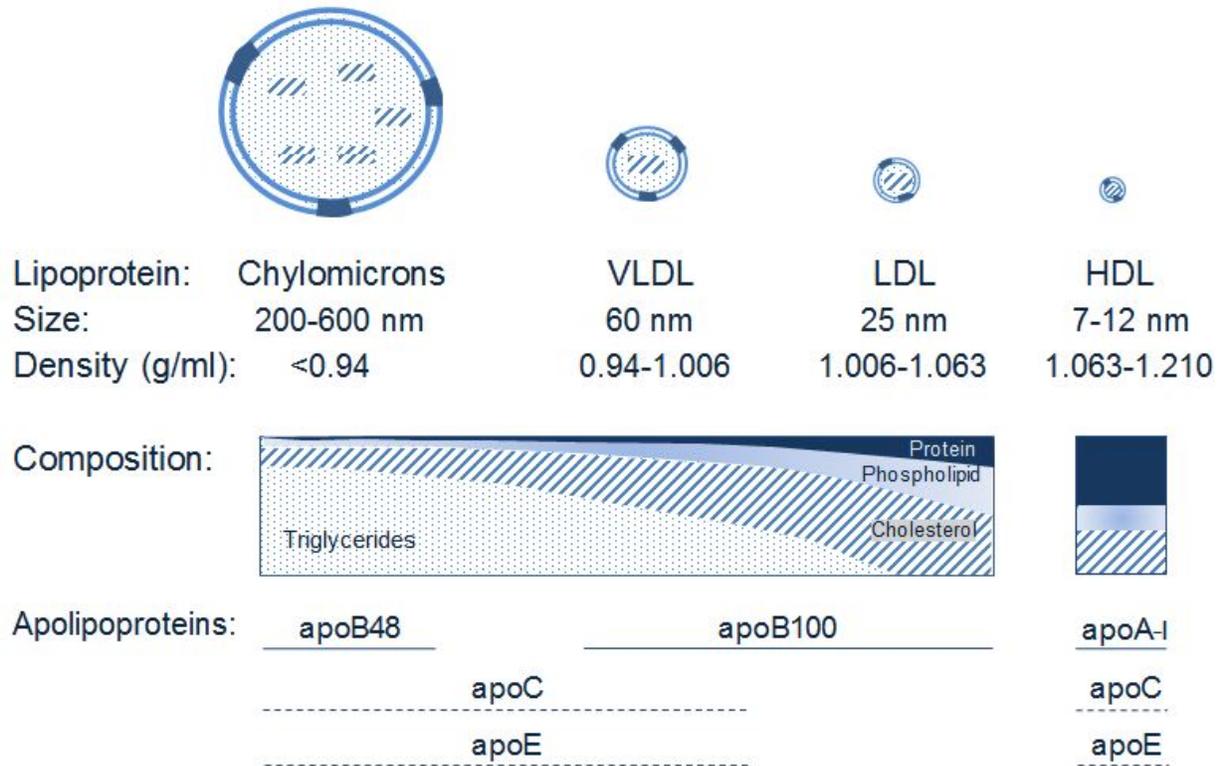


Figure 1.2: Schematic of the major peripheral lipoprotein classes.

The major apolipoprotein components of the lipoproteins are indicated with solid lines whereas dashed lines indicate secondary or minor apolipoprotein components

1.2.2 CNS lipoprotein and cholesterol metabolism

Cholesterol is highly enriched in the brain compared to other tissues. In most animal tissues, the average cholesterol concentration is 2mg/g tissue whereas its concentration is about 15-20mg/g tissue in the brain (Dietschy & Turley, 2004). While cholesterol is a critical component of neuronal membranes, the major pool of cholesterol in the CNS is in myelin that surrounds axons of the neurons that permits and facilitates the transmission of electrical signals. Myelin is provided by glial cells known as oligodendrocytes that are highly enriched in cholesterol. The rate of cholesterol synthesis in the brain is highest during development when active myelination

takes place (Spady & Dietschy, 1983). Though dramatically reduced in adult animals, cholesterol synthesis continues at a lower rate in glial cells (Nieweg et al., 2009).

All brain cells synthesize cholesterol during embryonic development, but mature neurons do not produce sufficient cholesterol to meet their lifelong requirements for membrane synthesis and repair (Dietschy & Turley, 2004). Adult neurons therefore depend upon lipids transported on glial-derived lipoproteins for optimal survival and maintenance. Glial cells are also critical for neurons to form numerous and highly efficient synapses (Nagler et al., 2001; Pfrieger & Barres, 1997; Ullian et al., 2001). In adult brains, CNS lipoprotein metabolism is particularly important after injury when lipid mobilization becomes critical for membrane-dependent processes such as reinnervation, synaptic remodeling and neuronal repair (Ignatius et al., 1987; Laskowitz et al., 1998; Poirier, 1994). The blood-brain-barrier separates the CNS from peripheral tissues, which does not permit entry of peripherally-derived cholesterol into the brain under normal circumstances (Bjorkhem & Meaney, 2004). The vast majority of CNS cholesterol is therefore synthesized *in situ* (Dietschy & Turley, 2001) and the CNS employs its own lipoprotein transport system that is separated from that of plasma, although many of the apolipoproteins involved in the peripheral lipoprotein metabolism, such as apoE, apoA-I, apoD and apoJ are also present in the brain. ApoB, on the other hand, is exclusively found in peripheral lipoproteins (J. E. Vance & Hayashi, 2010).

CNS lipoprotein particles resemble plasma HDL in density, size and composition (Ladu et al., 2000a; Pitas et al., 1987b). A major difference between brain and plasma HDL is that apoE is the major apolipoprotein in brain lipoproteins whereas apoA-I is the primary apolipoprotein on

plasma HDL (Asztalos, 2004; Ladu et al., 2000a; Pitas et al., 1987b). Although apoA-I is the most abundant apolipoprotein on peripheral HDL particles, in CSF, the concentration of apoA-I is only 0.5% of that in plasma (Pitas et al., 1987b). ApoA-I is not made within the brain but can, possibly in some species, be synthesized by endothelial cells that form the BBB (Balazs et al., 2004). ApoA-I is believed to have two routes of entry into the CNS, one being direct import across the BBB via an unidentified mechanism and the other being expressed by epithelial cells of the choroid plexus (Beffert et al., 1998). In contrast, the major CNS lipid carrier ApoE is abundantly synthesized and secreted by astrocytes (DeMattos et al., 2001) and also by microglia to a lesser extent (Gong et al., 2002). ApoE can also be synthesized in small amounts in the neurons in the hippocampus and cortex, but not in the cerebellum (P. T. Xu et al., 1999a). Some studies suggest that apoE is expressed at a low level in hippocampal neurons only in response to excitotoxic injury (P. T. Xu et al., 1999b; Q. Xu et al., 2006). Thus, neurons are only a quantitatively minor source of apoE in CNS. Because apoE does not cross the BBB, CNS apoE that is secreted by glial cells represents a distinct pool that does not intermingle with peripheral apoE, which is produced by hepatocytes and macrophages. This is evident in liver transplantation studies in humans, where the genotype of apoE in the plasma becomes that of the donor after transplantation, whereas the genotype of apoE in the CSF remains that of the recipient (Linton et al., 1991).

It is proposed that after being secreted by glial cells, apoE is assembled with phospholipids and cholesterol, which are synthesized by glia, into HDL-sized lipoprotein particles that can bind to apoE receptors expressed on neurons, thus delivering cholesterol to neurons for growth repair and synaptogenesis (H. Hayashi et al., 2004; Mahley, 1988; Pitas et al., 1998) (**Fig. 1.3**). To

maintain the cholesterol homeostasis in the CNS, excess cholesterol can be converted into 24(S)-hydroxycholesterol by the enzyme cholesterol 24-hydroxylase. This hydroxylase is restrictedly expressed in a subset of neurons and is essentially absent from astrocytes (Lund et al., 2003b). 24-hydroxycholesterol can readily cross the BBB and is delivered to the liver for further excretion into bile (Bjorkhem et al., 1998).

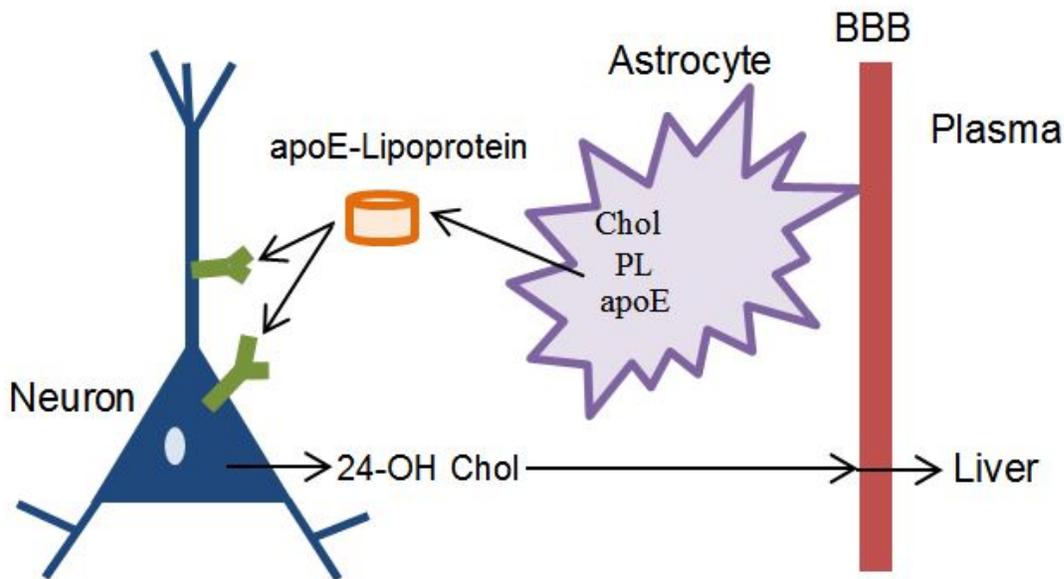


Figure 1.3: Schematic of cholesterol transport and homeostasis in the CNS.

Compared to plasma HDL lipoprotein metabolism, which is quite well-elucidated, many details regarding the formation, modification, regulation and function of apoE-containing CNS lipoprotein still remain to be fully understood. Many important proteins and enzymes that are critical for HDL metabolism, such as the ATP-binding cassette (ABC) sterol transporter family, lecithin:cholesterol acyltransferase (LCAT) and the LDL receptor (LDLR) family, are also found in the brain (Fryer et al., 2005a; Hirsch-Reinshagen et al., 2009), therefore similar mechanisms

have been proposed for CNS lipoprotein metabolism. Two poorly characterized mechanisms have been suggested for the formation of apoE-containing lipoprotein: (1) the direct secretion of lipidated apoE by glia, and/or (2) the secretion of lipid-poor apoE which is further lipidated extracellularly upon the efflux of cellular lipids. In plasma HDL formation, the ABC transporter ABCA1 is required for the initial acquisition of unesterified cholesterol and phospholipids by apoA-I (Bodzioch et al., 1999; Brooks-Wilson et al., 1999), which leads to the formation of nascent discoidal particles. These partially lipidated apoA-I particles subsequently accept more lipids from another ABC transporter ABCG1 (Kennedy et al., 2005; Nakamura et al., 2004; N. Wang et al., 2004). LCAT, the enzyme that converts unesterified cholesterol into cholesteryl esters, is required for the formation of mature spherical HDL particles. Previous work from our lab has established a critical role of ABCA1 in CNS lipoprotein formation, with more details to be discussed in Section 1.4. On the other hand, the role of ABCG1 in the brain is less clear. It is not yet known if a parallel sequence of events involved in formation of plasma HDL also operates for the formation of apoE-containing CNS lipoproteins. Although LCAT is expressed in the brain and glia-derived apoE particles can be substrates for LCAT (Hirsch-Reinshagen et al., 2009), the significance of this enzyme in brain lipoprotein metabolism still remain to be elucidated. This thesis further investigates the role of cholesterol transporter ABCA1 and apoE receptor LDLR in the formation, modification and regulation of apoE-containing CNS lipoprotein, and contributed to provide new knowledge to understand CNS lipoprotein metabolism.

ApoJ and apoD are other two apolipoproteins found in the CNS, although much less is known about these two apolipoproteins compared to apoE. ApoJ, also known as clusterin, is abundantly

expressed in the brain and secreted by astrocytes (Pasinetti et al., 1994), but are present on distinct populations of lipoprotein particles other than apoE (Q. Xu et al., 2000). Not much is known about the role of apoJ in CNS lipid metabolism and transport, but ApoJ has been reported to be a carrier of A β across the BBB (Bell et al., 2007; Zlokovic et al., 1996) and to suppress A β deposition in the brain (DeMattos et al., 2004; Nuutinen et al., 2009). ApoD could play a compensatory role for apoE as its expression level is found to be increased up to 50-fold in ApoE-deficient mice (Terrisse et al., 1999). Much more research needs to be done to understand the importance of these two apolipoprotein in CNS function.

1.3 ApoE in CNS function

ApoE plays a central role in the CNS. ApoE expression in glial cells increases by up to 150-fold after brain injury, consistent with the hypothesis that apoE-containing lipoprotein particles are involved in neuronal repair processes (A. M. Fagan et al., 1998; Ignatius et al., 1986; Snipes et al., 1986). Moreover, clearance of degenerating nerves after injury is impaired in apoE-deficient mice (A. M. Fagan et al., 1998). Also, apoE-deficient mice exhibit deficits on the hippocampus-dependent Morris water maze test (Gordon et al., 1995) as well as other learning deficits (Fullerton et al., 1998; Gordon et al., 1996; Masliah et al., 1995; Oitzl et al., 1997; Veinbergs et al., 1998), all suggesting a critical role of apoE in normal CNS functions.

1.3.1 ApoE isoforms

Human apoE is a 299 amino acid glycoprotein that exists in three major isoforms: apoE2, apoE3 and apoE4 that differ from one another in two amino acid positions, aa 112 and aa 158 (Weisgraber et al., 1994). ApoE contains two independently folded functional domains: The N-

terminal domain functions to bind apoE receptors (Siest et al., 1995) whereas the C-terminal domain binds lipids and interacts with other extracellular proteins such as A β (Pillot et al., 1999; Westerlund & Weisgraber, 1993) (**Fig. 1.4**). ApoE2 and apoE4 differ from apoE3 in a single amino acid. ApoE3 has cysteine (Cys) at residue 112 and arginine (Arg) at residue 158, whereas apoE2 has cysteine at both residues and apoE4 has arginine at both positions (Weisgraber, 1994). It has been identified that in apoE4 there exists a domain interaction between the glutamic acid at position 255 (Glu-255) in the C-terminal domain and the arginine at position 61 (Arg-61) in the N-terminal domain. This domain interaction is enhanced by the presence of Arg-112 in apoE4 but not by Cys-112 in apoE3 or apoE2 (Dong & Weisgraber, 1996; Mahley et al., 2006) (**Fig. 1.4**). Although like human apoE4, murine apoE has Arg at both position 112 and 158, instead of Arg-61, the apoE in all other species including rodents has threonine at position 61 (Thr-61) which prevents the domain interaction with Glu-255 (McLean et al., 1983)(**Fig. 1.4**).

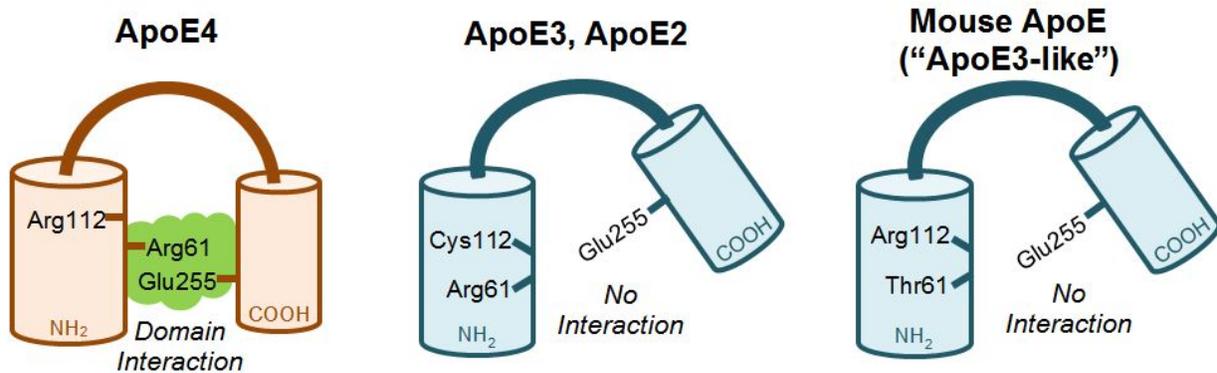


Figure 1.4: Schematic structures of rodent and human apoE isoforms.

In apoE4, Arg-61 in the N-terminal domain interacts with Glu-255 in the C-terminal domain. This domain interaction is promoted by Arg-112 in apoE4 but not by Cys-112 in apoE3 and apoE2. Mouse apoE, despite having Arg-112, has threonine at position 61 that does not participate in domain interaction therefore is functionally like apoE3.

Interestingly, when Thr-61 of murine apoE was replaced with arginine, the domain interaction was introduced which leads the Arg-61 mouse apoE to behave like human apoE4 (Raffai et al., 2001). These Arg-61 mice exhibit impaired astrocyte function and defects in synaptic transmission (Zhong et al., 2009). Therefore this domain interaction in apoE4 is believed to be the unique structural feature that is responsible for many of the detrimental effects of apoE in the neuropathology of various neurodegenerative disorders. Therapeutic strategies such as using small molecules to disrupt the domain interaction of apoE4 has also been proposed (Mahley et al., 2006). One hypothesis suggests that because of the domain interaction, apoE4 has a poorer ability to accept lipids, accounting for reduced lipid efflux from astrocytes to apoE4 than to apoE3. Michikawa et al. (2000) showed that in primary cultures of neurons and astrocytes, the addition of exogenous recombinant human apoE isoforms promoted the cholesterol efflux in an isoform-specific manner, potency in a rank order of apoE2>apoE3>apoE4. The authors also

reported that approximately twice as much cholesterol and phospholipid was released to apoE3 compared to apoE4 when the isoforms were expressed endogenously in mice lacking endogenous murine apoE. Consistent with these results, Minagawa et al. (2009) showed that 2.5- to 3.9-fold more cholesterol and phospholipids were effluxed from rat cortical astrocytes and neurons to recombinant human apoE3 than to apoE4. In addition, another recent study reported that the human apoE4 knock-in mice contained less amount of apoE in the brain than the human apoE3 knock-in mice did (Riddell et al., 2008). Furthermore, pulse-chase experiments in astrocytes revealed that the rate of degradation of apoE4 was greater than that of apoE3 (Riddell et al., 2008), suggesting that the poorly-lipidated apoE4 has a faster turnover in the cells that in turn may affect the intracellular fate of the proteins/receptors that could interact with apoE4. Nevertheless, the exact mechanisms explaining why apoE4 is detrimental in brain functions still remain as the subject of intense research.

1.3.2 ApoE receptors

Many members of the LDL receptor family are expressed in the CNS (reviewed in Holtzman et al. (2012)). For example, receptors, for which apoE is a high affinity ligand, such as the LDL receptor (LDLR), the LDL-receptor-related protein (LRP1), the VLDL receptor (VLDLR) and the apoE receptor-2 (apoER2) are expressed in neurons (Brown et al., 1997; D'Arcangelo et al., 1999; de Chaves et al., 1997; H. Hayashi et al., 2007; Herz, 2001; Posse De Chaves et al., 2000) as well as in astrocytes, microglia and oligodendrocytes (Q. W. Fan et al., 2001). Interestingly, only the LDLR appears to be up-regulated by cholesterol deficiency and down-regulated by cholesterol enrichment, indicating it may be the more important receptor specifically for lipid transport and maintaining cholesterol homeostasis in the brain (Q. W. Fan et al., 2001). Lipidated

apoE has a higher affinity to the LDLR than unlipidated or poorly-lipidated apoE does, also supporting this hypothesis (Ruiz et al., 2005).

It is proposed that the apoE-containing lipoprotein secreted and lipidated by astrocytes can bind to and be internalized by LDLR expressed on the cell membrane of neurons, thus delivering its lipid cargo to the neurons. Fryer et al. (2005a) showed that endocytosis of astrocyte-derived apoE-containing lipoproteins was impaired in brain cells that lacked the LDL receptor. CSF apoE levels in LDLR-deficient mice were 50% higher than in mice with normal LDLR levels (Fryer et al., 2005a). By contrast, in mice over-expressing LDLR in the brain, apoE levels were markedly reduced by 50-90% depending on the level of overexpression (J. Kim et al., 2009). Together these observations suggest that the LDLR is responsible for apoE endocytosis and plays an important role in regulating brain apoE levels.

Another receptor from the LDL receptor family, LRP1, is also highly expressed in neurons (Moestrup et al., 1992; Rapp et al., 2006; Rebeck et al., 1993). Interestingly, over-expression of LRP1 in the brain only modestly reduced the brain apoE levels in mice (Zerbinatti et al., 2006). It is found that LRP1 can bind to more than 30 ligands besides apoE, including APP, A β and α 2-macroglobulin, suggesting LRP1 does not act only as a receptor for endocytosis of lipoproteins but may also have critical roles in cell signalling. LRP1 is essential for early embryonic development, for LRP1-deficiency is lethal at the embryonic stage (Herz et al., 1992). Many recent studies have revealed that LRP1 can operate as a signaling receptor in regulating calcium signalling (Bacskai et al., 2000; Z. Qiu et al., 2003), long-term potentiation (LTP) (Zhuo et al., 2000), brain development (Herz & Chen, 2006), neurite growth (Holtzman et al., 1995), and

neuronal survival (Fumentalba et al., 2009; H. Hayashi et al., 2007, 2009). The specific role of LRP1 in A β clearance will be discussed in Section 1.3.4.2.

VLDLR and apoER2 are another two members from the LDL receptor family that are mainly expressed in neurons (Beffert et al., 2006; Posse De Chaves et al., 2000). In addition to their conventional roles as receptors for lipoprotein uptake, these two receptors are also key players in the Reelin signaling pathway that determines neuronal migration during brain development. The Reelin pathway is crucial for synaptic plasticity, dendritic spine development and LTP formation. Mice that lack both of these two receptors show severely impaired synaptic and neuronal functions (Beffert et al., 2004; Brandes et al., 2001; Niu et al., 2008; Stockinger et al., 1998; Trommsdorff et al., 1999).

In summary, apoE receptors from the LDL receptor family have a variety of physiological roles in CNS function. The primary function of these receptors was originally thought to be delivering lipids via endocytosis, yet it is now known that some of them also act as signaling receptors in CNS in response to the binding of apoE-containing lipoproteins or other signaling ligands.

1.3.3 ApoE in neuronal growth and synaptic function

Because neurons are extremely membrane-rich cells, neuronal regulation of cholesterol, which is mainly obtained from glia-derived apoE-containing lipoprotein, affects many aspects of neuronal function including neurite extension, vesicular transport, and synaptogenesis, particularly when this occurs as a function of new learning and memory (Dietschy & Turley, 2001) or during neuronal repair (Poirier, 1994). For example, traumatic brain injury (TBI) and experimentally

induced brain lesions lead to dramatic increases in apoE levels (T. Hayashi et al., 2006; Q. Xu et al., 2006), presumably to re-distribute lipids and strengthen apoE-mediated signaling for neuronal and synaptic repair.

Several studies demonstrate that apoE-containing lipoproteins stimulate neurite growth. When astrocyte-secreted apoE lipoproteins are supplied to primary cultures of rat retinal ganglion cells, axonal elongation is enhanced in a process that is mediated by a receptor of the LDLR family (H. Hayashi et al., 2004). Using β -VLDL enriched with apoE isoforms or apoE-containing HDL isolated from human CSF, apoE3 increases neurite outgrowth whereas apoE4 either decreases outgrowth or has no effect (A. M. Fagan et al., 1996; Holtzman et al., 1995; Nathan et al., 1994). Interestingly, in astrocytes isolated from mice lacking murine apoE but expressing human apoE isoforms, approximately twice as much cholesterol and phospholipid are effluxed to apoE3 compared to apoE4 (Michikawa et al., 2000). The inefficient delivery of lipids to neurons by lipid-poor apoE4-containing lipoprotein can be one explanation for the inability of apoE4 to promote neurite growth. However, possible roles of apoE isoforms in activating/suppressing signaling pathways underlying neurite growth should also be considered.

For many years, astrocytes were believed to play only a passive role in the CNS. However it is now evident that these cells actively contribute to neuronal excitability and synaptic transmission (Bezzi & Volterra, 2001; Fields & Stevens-Graham, 2002; Pfrieger, 2003; Vernadakis, 1996). For example, conditioned media from primary astrocyte-enriched cultures enhances synaptogenesis in cultured neurons (Pfrieger & Barres, 1997; Ullian et al., 2001), and the active component secreted by the astrocytes is the cholesterol contained in apoE-containing

lipoproteins (Mauch et al., 2001). Consistent with these findings, synaptic density is progressively reduced in an age-dependent manner in apoE-deficient mice (Masliah et al., 1995). This decrease is accompanied by disruptions to microtubules in dendrites, suggesting that these mice are unable to maintain necessary dendritic structures required for effective synaptic transmission (Masliah et al., 1995). Interestingly, mice lacking endogenous apoE but expressing human apoE3 are protected against this age-dependent neurodegeneration, whereas this protective effect is not seen in apoE4-expressing knock-in mice (Buttini et al., 1999). Instead, expression of human apoE4 in mice impairs synaptogenesis (White et al., 2001).

Memory and learning are impaired in apoE4 compared to apoE3 knock-in mice (Grootendorst et al., 2005; Raber et al., 1998; Raber et al., 2000), suggesting that isoform-specific apoE/apoE receptor-mediated lipid redistribution and signaling may play important roles in synaptic plasticity. Synaptic plasticity is defined as the ability of changing the connection (synapse) strength between neurons. Because memories are postulated to be represented by vast interconnected networks of synapses in the brain, synaptic plasticity is one of the important neurochemical foundations of learning and memory. Hippocampal LTP, a long-lasting enhancement in synaptic connections between two neurons that results from synchronous stimulation, is considered one of the major cellular mechanisms of memory storage (Bliss & Collingridge, 1993; Cooke & Bliss, 2006). ApoE may directly or indirectly affect synaptic plasticity, as apoE-deficient mice exhibit deficits on the hippocampus-dependent Morris water maze test (Gordon et al., 1995), and display impaired LTP in the CA1 region of hippocampus (Valastro et al., 2001). Interestingly, synaptic failure is also an early pathological feature of AD (Selkoe & Schenk, 2003). LTP in the hippocampus of apoE4 knock-in mice is significantly

impaired compared with apoE3 knock-in and WT mice (Trommer et al., 2004), suggesting a pathophysiological mechanism of apoE in AD that may involve its effects on synaptic functions. Importantly, what property of apoE4 results in its altered effect on synaptic plasticity is not well understood. Recently, apoE4 was found to selectively impair LTP by interfering with Reelin-induced phosphorylation and activation of the NMDA receptor, a voltage and ligand dependent glutamate receptor essential for LTP induction (Y. Chen et al., 2010). Reelin is a signaling ligand that activates various intracellular kinase pathways upon its binding to ApoER2 and VLDLR (Arnaud et al., 2003; Ballif et al., 2003; Beffert et al., 2005; Hiesberger et al., 1999; Weeber et al., 2002). This important study suggests that, because of its altered intracellular trafficking properties, apoE4 can impair Reelin-induced LTP by sequestering apoE receptors and glutamate receptors in intracellular compartments, thereby reducing their functional availability (Y. Chen et al., 2010).

1.3.4 ApoE in AD pathogenesis

After apoE was identified as a genetic risk factor for late-onset AD, the role of apoE in the pathogenesis of AD, especially its role in amyloid metabolism, has become an important subject under extensive research. Both apoE levels and apoE structure may influence its impact on A β and amyloid metabolism. Total loss of apoE in a transgenic mouse model of AD significantly reduces amyloid deposition without affecting A β production (Bales et al., 1997), suggesting that apoE may be involved in the conversion of soluble A β to its mature fibrillar form (Bales et al., 1999; Bales et al., 1997; Costa et al., 2004; Holtzman et al., 1999; Holtzman et al., 2000b; Irizarry et al., 2000). However, the relationship between apoE abundance and amyloid burden is far from simple. For example, apoE levels are increased by 50% in the brains of mice lacking the

LDL receptor as mentioned in the earlier section, yet, this has no impact on A β or amyloid load (Fryer et al., 2005a). Conversely, as described in more detail below, apoE levels are decreased by 75% in mice lacking the cholesterol transporter ABCA1, and this can be associated with increased amyloid deposition (Burns et al., 2006b; Hirsch-Reinshagen et al., 2005; R. Koldamova et al., 2005; Wahrle et al., 2005). Intriguingly, structural differences among human apoE isoforms and between murine and human apoE may also profoundly impact apoE and A β metabolism. Compared to human apoE2 and apoE3, apoE4 preferentially exists in a relatively unstable “molten globule” form due to its characteristic domain interaction that was discussed in section 1.3.1. AD mice expressing human apoE2, apoE3, and apoE4 in the absence of murine apoE develop the expected isoform-specific differences in amyloid load with apoE4 > apoE3 > apoE2, but the presence of human apoE considerably delays the onset of amyloid deposits compared with murine apoE (Buttini et al., 2002; Carter et al., 2001; Dodart et al., 2005; Holtzman et al., 2000a). Finally, the presence of the Dutch or Iowa mutations in A β causes primarily cerebrovascular amyloid deposits in humans (with human apoE) and in mice (with murine apoE). But replacement of murine apoE with either human apoE3 or apoE4 dramatically shifts the distribution of amyloid deposition from the cerebrovasculature to the parenchyma (F. Xu et al., 2008), a feature not observed in human subjects with the Dutch mutation. These observations suggest that the impact of apoE on brain physiology and A β metabolism is governed at many levels. Being an apolipoprotein, apoE exists in a variety of structural states depending on how much lipid cargo it carries. A growing body of literature demonstrates that the amount of lipids carried on apoE is a major determinant of A β and amyloid metabolism. Thus, understanding the nature of apoE-containing lipoproteins would provide significant insights to our understanding of the role of apoE in AD pathogenesis.

1.3.4.1 ApoE and A β interaction

ApoE is found co-localized in both parenchymal and cerebrovascular amyloid deposits (Burns et al., 2006a; M. P. Burns et al., 2003). Considering its ability to bind A β peptides, apoE has been suggested as a chaperone that modulates A β deposition and clearance. Residues 12-28 of A β have been identified as the binding site for apoE (Strittmatter et al., 1993b). On apoE, the binding site for A β is in the C-terminal domain, which overlaps with the lipid-binding site of apoE (Strittmatter et al., 1993b). Several studies have shown differences in the binding of apoE isoforms to A β . Some early in vitro studies found that purified recombinant apoE4 had a greater affinity for A β than apoE3, as these preparations of apoE4 binds A β in minutes, whereas apoE3-A β binding requires hours (Strittmatter et al., 1993b). However, opposite results were found when using apoE3 or apoE4 secreted by transfected eukaryotic cell lines, where the level of the apoE3-A β complex is 20-fold higher than that of the apoE4-A β complex (Ladu et al., 1994; Tokuda et al., 2000). It has also been shown that apoE3 associated with reconstituted HDL binds two to three fold more rapidly to A β than apoE4, indicating that the interaction of apoE and A β is also affected by the lipidation status of apoE (Tokuda et al., 2000). In vitro studies have demonstrated that apoE4 is more efficient than apoE3 at enhancing A β fibrillogenesis (Castano et al., 1995; Wisniewski et al., 1994). Although some in vitro studies using transfected cell lines suggest that apoE and its receptors may play roles in APP processing and A β production (Cam & Bu, 2006), and apoE4 has been found to enhance the synthesis of A β by promoting endocytic recycling of APP in some transfected cell lines (Ye et al., 2005), it is now generally believed that apoE is more likely to mediate A β clearance and the lipidation status of apoE plays a key role in this process. Two routes by which A β may be cleared in vivo include proteolytic degradation by

microglia and astrocytes within the brain (Cole & Ard, 2000; Koistinaho et al., 2004; Wyss-Coray et al., 2003), and transport across the BBB into the periphery mainly via LRP1 (Tanzi et al., 2004; Zlokovic, 2004).

1.3.4.2 ApoE and A β transport

Using state-of-the-art real time in situ microdialysis methods, Bell et al. (2007) demonstrated that free A β -40 is transported rapidly by LRP1 across the BBB at a rate six-fold faster than bulk interstitial fluid (ISF) flow, and that A β -40 has a faster rate of transport than A β -42. Association of A β -40 with lipid-poor apoE3 decreases its transport across the BBB. Furthermore, lipidated apoE virtually blocks all A β transport across the BBB over the 30 min time period used in the study. In contrast, apoJ promotes A β export across the BBB. Using the same in situ microdialysis methods, Deane et al. (2008) demonstrated that apoE disrupts the transport of A β from brain ISF in an isoform-specific manner, with apoE4 > apoE3 > apoE2. This study found that the binding of A β to apoE4 redirected the rapid export of free A β -40/42 from LRP1 to the VLDL receptor, which has a much slower interaction with A β -apoE complexes compared with LRP1, resulting in increased brain retention of apoE-A β 40/42 complexes by 15- and 9-fold, respectively, compared with the free peptides. On the other hand, apoE2 and apoE3 and their complexes with A β were transported across the BBB via both VLDLR and LRP1 at a substantially faster rate than apoE4 complexes. Consistent with the Bell study, Deane et al. (2008) also showed that lipidation of all three apoE isoforms (secreted by immortalized mouse astrocytes expressing human apoE) greatly reduced the amount of apoE and apoE-A β complexes that were transported across the BBB (**Fig. 1.5**). The above findings suggest that the reduction of fibrillar A β deposits in apoE-deficient AD mice may in part be due to improved A β transport

across the BBB due to loss of apoE-mediated A β retention in the brain. The isoform-specific disruption of free A β transport may also in part explain the greater amyloid burden in AD mice expressing human apoE4 than apoE2 or apoE3.

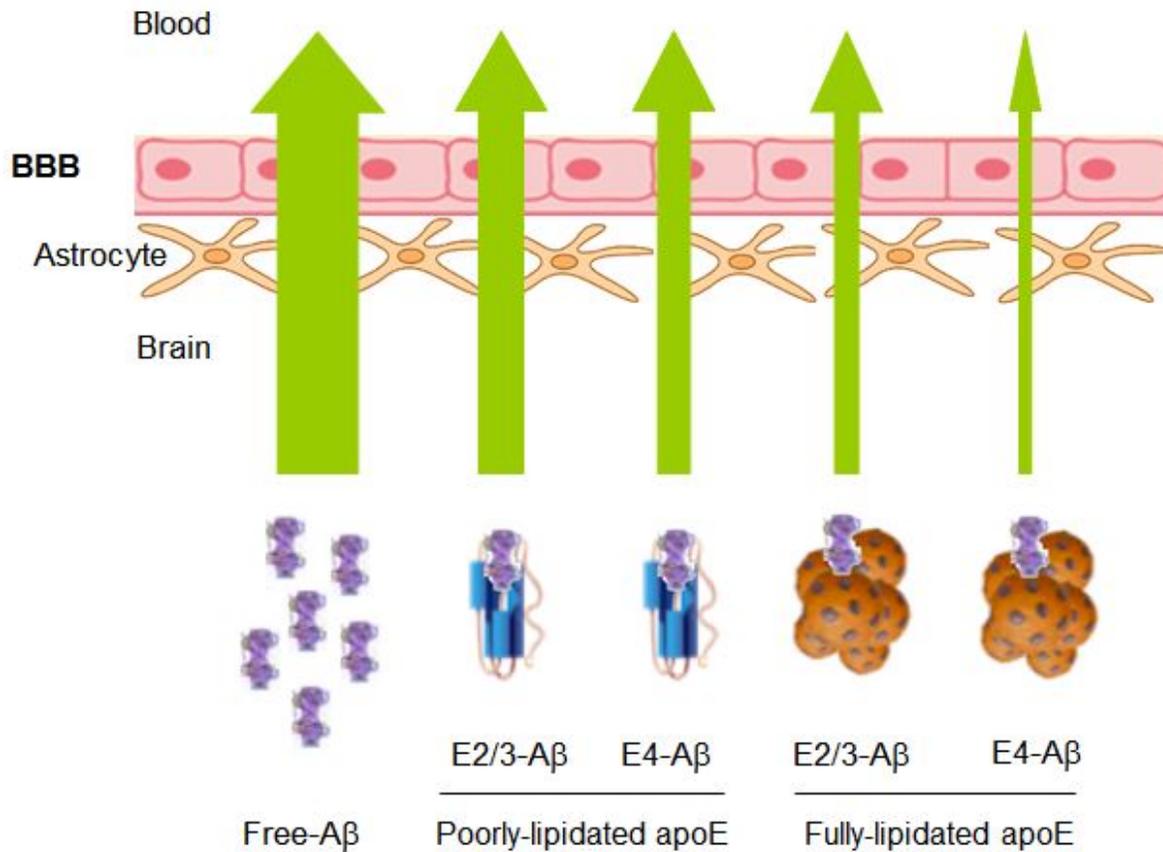


Figure 1.5: Effect of apoE on A β transport across the BBB.

Free A β is efficiently transported across the BBB, but this process is slowed in the presence of apoE in an isoform specific manner, with apoE4 inhibiting A β transport more than apoE2 or apoE3. Lipidated apoE inhibits A β transport even more, again according to apoE isoform.

1.3.4.3 ApoE and A β degradation

Another route by which A β may be cleared in vivo is through proteolytic degradation. Receptor-mediated endocytosis of the apoE-A β complex could lead A β to be delivered to the lysosomes for intracellular degradation. Neprilysin is one important proteolytic enzyme that degrades A β peptides intracellularly (Iwata et al., 2000). There also exist extracellular proteolytic enzymes, such as insulin-degrading enzyme (IDE) that mediates the degradation of A β peptides in the extracellular milieu (Kurochkin & Goto, 1994; W. Q. Qiu & et al., 1998). Neprilysin is highly expressed in microglia (Iwata et al., 2000; Rogers et al., 2002), and IDE is secreted by both astrocytes and microglia (Kurochkin & Goto, 1994). Inhibition of these proteinases (Dolev & Michaelson, 2004) or silencing of their genes (Iwata et al., 2000) results in a significant increase of brain A β levels and promotes plaque deposition. In contrast, overexpressing IDE or neprilysin lowers brain A β levels and eliminates plaque formation (Hemming et al., 2007). Given that A β 42 is less efficiently exported through the BBB compared with A β 40 (Bell et al., 2007; Deane & et al., 2004), the removal of A β 42 via proteolytic degradation may be a key determinant of A β and amyloid homeostasis in the brain.

Q. Jiang et al. (2008) reported that apoE acts to facilitate the proteolytic degradation of A β and its lipidation status is a critical determinant of its ability to stimulate both intracellular A β degradation by neprilysin and extracellular A β degradation by IDE. Microglia cells efficiently take up and degrade soluble A β , and this process is facilitated if A β is added together with human apoA-I or apoE purified from plasma. In this assay, human apoE2 promotes maximal A β degradation, whereas apoE4 is the least efficient, consistent with the effect of apoE genotype in human AD. Importantly, loss of either of apoE or ABCA1 (the cholesterol transporter) activity

impairs the ability of primary microglia to degrade A β . Conversely, enhanced ABCA1 expression by liver-X-receptor (LXR) agonist stimulates A β degradation. This study has defined that both apoE isoform and its lipidation status influences its capacity to promote A β degradation.

The above sections discussed two major pathways by which the lipidation status of apoE may affect A β clearance: (1) regulation of A β transport across the BBB (**Fig. 1.5**), and (2) regulation of A β proteolytic degradation by neprilysin and IDE. The relative contribution of these pathways to overall A β clearance in vivo is a key question to address. For example, the rapid transport of A β and lack of significant cellular A β degradation within the brain under the in situ microdialysis conditions used by Bell et al. (2007) and Deane et al. (2004) suggests that A β efflux across the BBB may be the major mechanism of A β clearance with proteolytic degradation playing only a minor role. However, it is worth noting that these microdialysis experiments are completed over a 30-300 min time period and trace an exogenous bolus of labeled A β or preformed A β -apoE complexes injected into the animal's brain, which may be metabolized differently than endogenous A β . Furthermore, several studies have shown that LXR agonists facilitate apoE lipidation but reduce A β levels (Eckert et al., 2007; Q. Jiang et al., 2008; Lefterov et al., 2007; Riddell et al., 2007). These findings contradict what the microdialysis experiments would predict - that LXR agonists might increase brain levels of A β because they enhance the lipidation of apoE. Exactly how lipidated apoE regulates A β homeostasis has yet to be fully understood.

More recent studies provide evidence for perivascular drainage to be a third pathway of A β removal. Extracellular fluid is drained from the brain towards the cervical lymph nodes via perivascular channels. One group showed that large amounts of A β could be found within the perivascular space of 25 months old APP-transgenic mice, whereas only small amounts of A β could be detected in the perivascular space of wild-type mice, suggesting that altered drainage along perivascular channels may also contribute to the A β accumulation and deposition in AD brain (Thal et al., 2007). Moreover, a 2012 study using targeted replacement mice expressing the human apoE3 and apoE4 genes showed that arterial perivascular drainage of A β from the brain was disrupted in human apoE4-expressing mice (Hawkes et al., 2012). After mice received intracerebral injections of human A β 40, aggregation of A β 40 in periarterial drainage pathways was found only in apoE4-expressing mice, but not in apoE3-expressing or wild-type mice, suggesting that apoE4 may increase the risk for AD through disruption and impedance of perivascular drainage of soluble A β from the brain (Hawkes et al., 2012). Nevertheless, the specific roles of apoE and CNS lipoproteins in this drainage pathway remain largely unstudied.

1.4 ABCA1: a key regulator of cholesterol metabolism

Identified and cloned in 1994, ABCA1 belongs to subfamily A of a large superfamily of ATP-binding cassette (ABC) transmembrane transporters (Luciani et al., 1994). ABCA1 gene encodes a 2261 amino acids (254kDa) integral transmembrane protein that consists of two halves of similar structure. Each half has a transmembrane domain containing six helices followed by a cytoplasmic nucleotide binding domain (M. Dean et al., 2001). ABC transporters derive energy from ATP molecules to drive the transport of different substrates across cell membranes (Michael Dean et al., 2001). In particular, ABCA1 transports cholesterol and phospholipids

across the plasma membrane onto poorly lipidated apoA-I, which mediates the rate-limiting step of HDL biogenesis (Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Rust et al., 1998; Tall & Wang, 2000).

1.4.1 ABCA1 in HDL biogenesis

The critical role of ABCA1 in HDL biogenesis was discovered when ABCA1 was identified as the product of the defective Tangier Disease (TD) gene (Bodzioch et al., 1999; Brooks-Wilson et al., 1999). Defective mutations in the heterozygous forms of ABCA1 gene cause familial hypoalphalipoproteinemia and cause TD in the homozygous forms. Patients of both conditions exhibit impaired cellular cholesterol efflux, highly inefficient reverse cholesterol transport (RCT) and low levels of plasma HDL particles. TD patients also present with extremely low plasma apoA-I levels and deposition of intracellular cholesterol esters in the reticuloendothelial system with hepatosplenomegaly and enlarged tonsils with yellowish-orange color. These observations clearly show that regulating HDL metabolism is the primary biochemical function of ABCA1. Phenotypes observed in both ABCA1-deficient and overexpressing animal models also support this idea. Like TD patients, ABCA1 knockout animals exhibit dramatic decrease in total and HDL-cholesterol levels, hypercatabolism of apoA-I and accumulation of cholesteryl esters in macrophage-rich tissues (Christiansen-Weber et al., 2000; McNeish et al., 2000; Timmins et al., 2005). In contrast, ABCA1-overexpressing mice have elevated plasma HDL, increased circulating apoA-I levels and are protected from macrophage lipid accumulation (Joyce et al., 2002; Singaraja et al., 2002; Wellington et al., 2003).

Although it is well established and unquestioned that ABCA1 is critical for plasma HDL biogenesis and mediates cellular cholesterol efflux, the exact mechanisms by which ABCA1 achieves these effects are yet to be fully understood. Two models have been proposed for how ABCA1 effluxes lipids on to apolipoproteins. In one proposed model, apoA-I binds ABCA1 at the cell surface and is subsequently internalized and delivered to late endosomes where apoA-I receives lipids and then the lipid-apolipoprotein complexes are then re-secreted by exocytosis (W. Chen et al., 2005). Another more popular model hypothesizes that the process starts with the binding of a small pool of apoA-I to the extracellular loop of ABCA1 that stabilizes the transporter at the cell surface, inhibits its degradation and upregulates its activity. The increased translocase activity of ABCA1 thus flip excess cholesterol and phospholipids from the cytosolic leaflet of the plasma membrane to the external leaflet, generating a propitious membrane microenvironment for more apoA-I docking with high affinity. Eventually the bound apoA-I spontaneously solubilizes the membrane phospholipids and cholesterol in the microenvironment, followed by formation of discoidal HDL particles with two, three or four apoA-I molecules per particle (Chambenoit et al., 2001; Oram & Heinecke, 2005; Vaughan & Oram, 2003; Vedhachalam et al., 2007). These two distinct mechanisms, however, are not necessarily mutually exclusive although there is a controversy with regard to which one is dominant. It is believed that ABCA1-mediated lipidation of apoE follows similar mechanism in the CNS, though much more research needs to be done to elucidate the exact mechanisms.

1.4.2 ABCA1 in brain lipoprotein metabolism

While the role of ABCA1 in HDL biogenesis and apoA-I metabolism is well established, its role in CNS cholesterol and lipoprotein metabolism has only recently started to be appreciated. As

early as in 1994, Luciani et al. discovered a relatively high expression of ABCA1 in adult mouse brain. However during the next following 10 years, the transporter did not attract much attention from neuroscientists. Whitney et al. (2002) provided first evidence that LXRs regulate lipid homeostasis in glial cells by transcriptionally regulating the expression of key proteins that are involved in cholesterol efflux and synthesis, including ABCA1, ABCG1 and sterol regulatory element-binding protein 1 (SREBP1). Koldamova et al then published brain region and cell type specific expression of rat ABCA1 in 2003. The study also showed that LXR ligand treatment of embryonic neurons and astrocytes not only increased expression of ABCA1 but also elevated apoA-I- and apoE-mediated cholesterol efflux. These observations suggest that ABCA1 could as well play an important role in CNS lipoprotein biogenesis.

Work from our laboratory and that of the Holtzman group has further established that ABCA1 plays a key role in moving cholesterol onto CNS apoE, in regulating glial apoE secretion, and in determining apoE levels in brain tissue (Hirsch-Reinshagen et al., 2004; Wahrle et al., 2004). In mice, ABCA1 deficiency leads to a dramatic 80% decrease in apoE levels in brain tissue, cerebrospinal fluid (CSF), and plasma, whereas apoJ levels are not affected (Hirsch-Reinshagen et al., 2004; Wahrle et al., 2004). ABCA1-deficient astrocytes and microglia are impaired in their ability to efflux cholesterol to apoE (Hirsch-Reinshagen et al., 2004) and secrete poorly-lipidated apoE particles compared with wild-type glia (Wahrle et al., 2004). In the CSF of ABCA1^{-/-} mice, apoE-containing lipoprotein particles are dramatically reduced in abundance and contain an abnormally small species (Wahrle et al., 2004). Taken together, these results suggest that poorly lipidated apoE may be degraded more quickly in the brain, similar to the rapid renal catabolism of poorly lipidated plasma apoA-I. Notably, apoE levels are unchanged in the

absence of the related cholesterol transporter ABCG1, which is highly expressed in brain (Burgess et al., 2008). These observations support the hypothesis that ABCA1 is the major molecule that lipidates apoE and affects its turnover in the brain, similar to the effects of ABCA1 on peripheral apoA-I lipidation and catabolism.

1.4.3 ABCA1 and AD

Whether the prevalence of AD is increased in TD patients has not been addressed, primarily because fewer than 100 TD subjects have ever been documented and most do not survive past 70 years of age (Fredrickson et al., 1961). However, there is a single case report of a TD patient who died of cerebral amyloid angiopathy, which is characterized by A β deposition in cerebral blood vessels, a pathology that is present in 80-90% of AD patients (Ghiso & Frangione, 2001; Grammas et al., 2002; Hong et al., 2002; Jellinger, 2002). Whether ABCA1 polymorphisms are genetically associated with AD remains an open question. Some studies suggest that ABCA1 variants affect CSF A β or cholesterol levels, risk, or age of onset of AD (Katzov et al., 2006; Katzov et al., 2004; Sundar et al., 2006; Wollmer et al., 2003), whereas others find no association (Kolsch et al., 2006; Y. Li et al., 2004). One study suggests an association only in relation to APOE ϵ 4 carriers (Shibata et al., 2006).

On the other hand, in vivo animal studies showed that ABCA1 can be a major determinant of amyloid burden. Three independent groups simultaneously crossed ABCA1-deficient mice to a total of four independent murine models of AD to determine the impact of ABCA1 on amyloid burden, A β levels, and apoE levels (Hirsch-Reinshagen et al., 2005; R. Koldamova et al., 2005; Wahrle et al., 2005). All of these studies found that elimination of ABCA1 had no significant

impact on APP processing or steady state A β levels. These observations suggest that the impaired ability to efflux cholesterol does not greatly affect A β production in vivo. In contrast, each study confirmed the 70–80% reduction in apoE levels in the absence of ABCA1. Because apoE is required for amyloid deposits to form in mice (Bales et al., 1999), each group predicted that amyloid plaque burden would be correspondingly reduced in the absence of ABCA1. However, amyloid load was undiminished in each of the four models examined, and, in contrast to expectations, was significantly increased in two of the models. These data provided clear support for the impact of ABCA1 on amyloidogenesis by mechanisms independent of A β production.

Contrasting the detrimental effect of ABCA1 deficiency on amyloid burden, Wahrle et al. (2008) demonstrated that overexpressing ABCA1 by six fold or more in the brain promotes the formation of lipid-enriched CSF apoE lipoproteins and virtually eliminates the formation of mature amyloid plaques in vivo without altering APP processing. These findings demonstrate that excess ABCA1 is sufficient to mitigate amyloid deposition in vivo, most likely by promoting apoE-mediated A β clearance. Notably, the protective effects of excess ABCA1 are observed only on achieving a threshold of ABCA1 overexpression by six fold or greater in vivo (Hirsch-Reinshagen et al., 2007; Wahrle et al., 2008). Interestingly, increased lipidation of apoE in these mice also drives the cellular uptake of brain lipoproteins via apoE receptors and leads to a net 30% reduction of apoE levels in brain tissue. These results suggest that the amount of lipid carried on apoE may be a more important functional parameter for A β metabolism and amyloidogenesis in the brain than total apoE levels. The outcomes of these in vivo studies using

both ABCA1-deficient and ABCA1-overexpressing animal models strongly supports the idea that ABCA1-mediated lipidation of apoE facilitates the clearance of A β peptides.

A recent paper from the Koldamova laboratory further demonstrated that ABCA1-deficiency specifically affected AD pathology in human apoE4 but not in human apoE3-targeted replacement mice (Fitz et al., 2012). In this study, the authors generated AD mice that express human apoE3 or apoE4 (APP/E3 and APP/E4 respectively) by crossing APP/PS1 Δ E9 mice with human apoE3- and apoE4-targeted replacement mice. The APP/E3 and APP/E4 mice were then crossed with ABCA1-KO mice. Due to the extensive breeding challenges of this project, the authors generated APP/E3 and APP/E4 mice that express wild-type ABCA1 or ABCA1 hemizygous (APP/E3/ABCA1-/+ and APP/E4/ABCA1-/+). Nevertheless, the authors found that lack of one gene dose of ABCA1 affected cognitive performance in an apoE isoform dependent manner. Specifically, APP/E4/ABCA1-/+ mice performed significantly worse than APP/E3/ABCA1-/+ mice in both radial water maze and contextual fear conditioning test, whereas no difference was found between APP/E4 and APP/E3 mice. Amyloid load, soluble A β level and insoluble A β level were all increased by ABCA1 hemizyosity in APP/E4 but not in APP/E3 mice. In vivo microdialysis analysis showed that A β level in ISF and A β half-life were increased in APP/E4 mice and were even higher in APP/E4/ABCA1-/+ mice, suggesting that A β clearance from CNS was further worsened by ABCA1 deficiency in APP/E4 mice. Interestingly, plasma HDL and plasma A β levels in APP/E4/ABCA1-/+ mice were significantly decreased and a negative correlation between plasma HDL and amyloid plaques in brain was found, suggesting

that plasma lipoproteins may also be involved in A β clearance in these mice. Overall, this study further highlights the importance of ABCA1-apoE pathway in AD pathogenesis.

1.5 LXR: a master regulator of lipid homeostasis

1.5.1 Overview

Both ABCA1 and apoE are target genes of the nuclear receptor liver X receptor (LXR) (Costet et al., 2000; Schwartz et al., 2000; Venkateswaran et al., 2000). Nuclear receptors are master regulators of transcriptional programs that integrate the homeostatic control of many biological processes. LXR exists as two isoforms, LXR α (447 amino acids) and LXR β (460 amino acids). Both isoforms have considerable sequence homology and share approximately 78% identical amino acid sequence at the DNA- and ligand-binding domains (Zelcer & Tontonoz, 2006). LXR α and LXR β can be activated by the same ligands and also have almost identical targets (Fu et al., 2001; Janowski et al., 1996). However, the two isoforms differ in their expression levels from tissue to tissue. For example, LXR α is highly expressed in the liver, adipose tissues, adrenal glands, intestine, kidney and macrophages, whereas LXR β is ubiquitously expressed. LXR α is the predominant isoform that regulates cholesterol metabolism in liver (Beltowski & Semczuk, 2010; Jamroz-Wisniewska et al., 2007), whereas LXR β is the main isoform found in the CNS (Whitney et al., 2002).

The natural ligands for LXR include oxidized derivatives of cholesterol known as oxysterols (Janowski et al., 1996; Lehmann et al., 1997). Oxysterols produced by enzymatic reactions are potent LXR agonists and can be divided into three groups:

- (1) intermediates of the cholesterol biosynthetic pathway, i.e. 24(S),25-epoxycholesterol in the liver (Janowski et al., 1996);
- (2) intermediates in the synthesis of steroid hormones from cholesterol, i.e. 22(R)-hydroxycholesterol and 20(S)-hydroxycholesterol (Janowski et al., 1996);
- (3) cholesterol metabolites produced by sterol hydroxylases, i.e. 24(S)-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol (Bjorkhem et al., 1994; Lin & Smith, 1974; Lund et al., 1998).

Synthetic compounds including TO901317 (Tularik) (Schultz et al., 2000) and GW3965 (Lund et al., 2003a) that activate both LXR α and LXR β have been developed for preclinical in vivo use. TO901317 activates both LXR α and LXR β equally, whereas GW3965 has a greater affinity toward LXR β than LXR α (Lund et al., 2003a).

LXRs generally function as heterodimers with retinoid X receptor (RXR) (Claudel et al., 2001). RXR is also a common partner for several other nuclear receptors including the retinoid acid receptor, peroxisome proliferator-activated receptor (PPAR) and thyroid hormone receptor (Chawla et al., 2001). LXR/RXR complexes can be activated by LXR agonists or 9-*cis*-retinoic acid, an endogenous RXR agonist. LXR/RXR heterodimers bind to a specific DNA sequence known the LXR response element (LXRE) in the promoter region of target genes, thus either repressing or activating gene expression. LXR can regulate gene expression by three different mechanisms: (1) ligand-independent repression, (2) ligand-dependent transactivation, and (3) ligand-dependent transrepression (**Fig. 1.6**). When no ligand binds to the LXR/RXR complex, the heterodimer binds to co-repressors, together suppressing the transcriptional activity of target genes. Upon binding to the ligand, LXR/RXR heterodimer becomes dissociated with the co-

repressors and instead recruits co-activators, thus leading to the transcription of target genes. In the third model, binding to the ligand results in LXR being coupled with a member from the small ubiquitin-like modifier (SUMO) family, a process known as LXR SUMOylation (Ghisletti et al., 2007). LXRs that are activated by natural or synthetic agonists conjugate to SUMO2/3. The LXR-SUMO2/3 complex is then recruited to nuclear factor κ B (NF- κ B) promoter, preventing nuclear receptor co-repressor 1 (NCoR) from being removed, eventually resulting in inhibition of transcription of those proinflammatory genes that do not contain LXRE (**Fig. 1.6**).

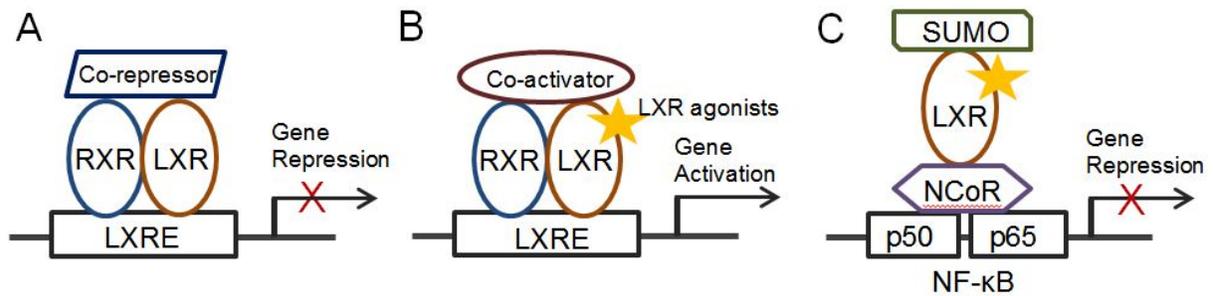


Figure 1.6: Three mechanisms of LXR-regulated gene expression.

(A) In the absence of ligands, LXR/RXR complexes bind to LXRE and recruit co-repressors leading to the suppressed transcription of target genes. (B) Once binding to the ligands, co-repressors are dissociated from LXR/RXR heterodimers which in turn bind to co-activators, leading to the activation of target gene expression. (C) Binding ligands to LXR can result in LXR binding with SUMO. SUMO-LXR complex is then recruited to NF- κ B promoter, securing co-repressor NCoR, thus inhibiting NF- κ B-dependent activation of proinflammatory gene expression.

Over the last decade, key roles of LXR in lipid metabolism, glucose homeostasis and inflammatory signaling have been established. Activation of LXR induces the expression of target genes that have functions in (1) cholesterol trafficking and efflux, such as ABCA1 and ABCG1 that participate in the removal of excess lipids from cells (Oram & Heinecke, 2005; Vaughan & Oram, 2005; N. Wang et al., 2004); (2) lipoprotein remodeling, for example ApoE, lipoprotein lipase, cholesteryl ester transfer protein (CETP), and phospholipid transfer protein (PLTP) that regulate the lipoprotein composition; (3) lipogenesis, including sterol regulatory element binding protein 1c (SREBP1c), fatty acid synthase (FAS), stearoyl coenzyme A desaturase (SCD), and acyl coenzyme A carboxylase. On the other hand, LXR also down-regulates genes involved in cholesterol biosynthesis, including lanosterol 14 α -demethylase (CYP51A1), and squalene synthase (farnesyl diphosphate farnesyl transferase 1, FDFT1) (Y. Wang et al., 2008). LXRs also inhibit a set of inflammatory genes normally activated in

response to bacterial, LPS, TNF- α , or IL-1 β stimulation (Lefterov et al., 2007; Zelcer & Tontonoz, 2006).

Within the periphery, LXRs are cholesterol sensors that sense excess cholesterol and trigger various adaptive mechanisms to protect cells from cholesterol overload. The activation of LXRs stimulates RCT, i.e. the removal of excess cholesterol by ABC transporters (primarily ABCA1 and ABCG1) onto cholesterol carriers (including apoA-I and apoE), followed by transport of this cholesterol to the liver for biliary excretion (Kapur et al., 2008). LXR activation also suppresses intestinal cholesterol absorption (Repa & Mangelsdorf, 2000), and inhibits cellular cholesterol synthesis and uptake (Schultz et al., 2000). Within the CNS, several studies have shown that LXR agonists increase the expression of typical target genes including ABCA1, ABCG1, and apoE in astrocytes, suggesting LXRs may also regulate brain cholesterol balance (Fujiyoshi et al., 2007; Liang et al., 2004; Whitney et al., 2002).

Functional anti-inflammatory effects of LXRs have been confirmed in vivo. Fowler et al. (2003) demonstrated that LXR activation led to a marked decrease in 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced cutaneous inflammation in oxysterol treated mice compared to vehicle treated animals. Terasaka et al. (2005) showed that treatment of mouse peritoneal macrophages with an LXR agonist reduced the expression of tissue factor following induction of proinflammatory stimuli including lipopolysaccharide (LPS). Tissue factor is expressed by macrophages and other types of cells within atherosclerotic lesions and plays an important role in thrombus formation. Intriguingly, the combined positive effects of LXRs on macrophage RCT and inflammation are anti-atherogenic, as treatment of atherosclerosis-prone apoE^{-/-} and LDLR-

/- mice with a synthetic LXR ligand lead to a 50% reduction in lesion size (Joseph & et al., 2002). Conversely, macrophage-specific loss of LXRs achieved by transplantation of bone marrow from LXR $\alpha\beta$ -/- mice into either apoE-/- or LDLR-/- mice markedly increases atherosclerotic lesion size (Tangirala et al., 2002). Because inflammatory processes also contribute to the initiation and progression of AD, the combinatorial benefits of LXRs are of growing interest for their possible application to AD.

1.5.2 Potential therapeutic for AD

Both TO901317 and GW3965 cross the BBB and stimulate transcription of LXR target genes including ABCA1 and apoE in the brain. Several studies have demonstrated that these synthetic agonists reduce A β levels and improve behavioral deficits in AD mouse models (Eckert et al., 2007; Q. Jiang et al., 2008; Lefterov et al., 2007; Riddell et al., 2007). Specifically, extended GW3965 treatment in the Tg2576 AD mouse model led to a ~50% reduction in plaque number and a 67% reduction in plaque load in the hippocampus, as well as a 50% reduction in both A β 40 and A β 42 levels along with an increase in brain levels of apoE and ABCA1 (Q. Jiang et al., 2008). Similar findings were also reported by Riddell et al. (2007) who observed a significant reduction in hippocampal A β 42 but not A β 40 levels as well as an expected increase in ABCA1 and apoE mRNA when Tg2576 AD mice were treated with TO901317. It was also demonstrated that there was an improvement in the contextual memory deficit that is evident in the Tg2576 AD mouse model following treatment of LXR agonists (Q. Jiang et al., 2008). GW3965 also improved the ability of microglia to clear A β via neprilysin and IDE, and this effect is dependent on both ABCA1 and apoE (Q. Jiang et al., 2008). In addition, LXR agonist treatment showed an increase in soluble apoE and apoA-I and a decrease of insoluble A β (Lefterov et al., 2007).

Finally, in vivo treatment with TO901317 was found to reduce cholesterol levels in neuronal membranes of the CNS along with an upregulation of ABCA1, ABCG1, and apoE (Eckert et al., 2007). Taken together, these studies suggest that agents such as LXR agonists may provide a novel therapeutic strategy for AD treatment. But there remain some unanswered questions as whether sustained use of LXR agonists may have prophylactic and/or therapeutic benefit for cognitive and neuropathological outcomes for AD and whether ABCA1 is the primary LXR-target gene that mediates the beneficial effects of LXR agonists.

1.5.3 Problems with current LXR agonists

Although the synthetic LXR agonists TO901317 and GW3965 are effective in preclinical studies in AD mice, all known LXR agonists have a significant caveat that precludes their translation into human clinical trials. Specifically, activation of FAS and SREBP-1c transcription in the liver leads to hypertriglyceridemia and hepatic steatosis (fatty liver), a detrimental side effect that is particularly problematic in species such as humans that express CETP (Groot et al., 2005). Despite significant medicinal chemistry efforts by many pharmaceutical companies, it has not yet been possible to develop a compound that stimulates beneficial LXR-responsive pathways without also activating hepatic FAS and SREBP-1c. As a result, the therapeutic potential of these agonists for AD remains untapped.

1.6 Summary, hypothesis and specific objectives

Lipoproteins in the CNS resemble circulating HDL particles in size and density. The major difference between brain and peripheral HDL is that apoE is the major protein of CNS lipoproteins, whereas the principal protein component of peripheral HDL is apoA-I. Glial cells,

including astrocytes and microglia, are the major source of CNS apoE. ApoE plays a pivotal role in lipid and lipoprotein metabolism and is involved in pathogenesis of neurodegenerative diseases such as Alzheimer's disease. ABCA1 effluxes cholesterol and phospholipids to apolipoprotein acceptors including apoE. We have previously shown that ABCA1 is a key regulator of apoE levels and lipidation in the brain and that deficiency of ABCA1 increases amyloid burden in AD mouse models. Translating these results into potential therapies for AD, however, will require a more thorough understanding of the biochemical nature of nascent apoE particles generated from wild-type (WT) and ABCA1^{-/-} glia and of lipoprotein remodeling in CNS in general. In non-CNS cells, approximately 70% of secreted apoE taken up by the LDLR pathway is recycled (S. Fazio et al., 1999; S. Fazio et al., 2000; Rensen et al., 2000). ApoE recycling has been shown to be stimulated by HDL and apoA-I and linked to cholesterol efflux, but whether similar pathways exist in the brain and involve ABCA1 has not been addressed. Understanding these pathways may lead to novel ways to augment apoE secretion or to rescue impaired apoE secretion/lipidation to protect the brain from chronic and acute damage, as the detrimental form of apoE for AD, apoE4, has recently been shown to be impaired in its recycling. ABCA1 and apoE gene expression are regulated by LXR. Synthetic LXR agonists have been shown to facilitate A β clearance possibly via enhancing ABCA1-mediated apoE lipidation. However, current synthetic LXR agonists such as TO901317 and GW3965 also cause undesired side effects such as hypertriglyceridemia and hepatic steatosis. The field is therefore extremely interested in identifying apoE modulators that may enhance apoE secretion and lipidation without exerting adverse side effects.

The overall hypothesis of this thesis is that **ABCA1 plays an essential role in regulating glial apoE secretion, lipidation and recycling**. The main goal of this thesis is to investigate the pathways and mechanisms of how apoE-containing CNS lipoprotein is remodeled via secretion, lipidation and recycling, and how apoE is regulated by several steroidal modulators, and particularly the role of ABCA1 in these processes. In this thesis, vitro cell models were the main platform to study this hypothesis. The hypothesis is being tested using the following specific objectives:

Aim 1: To elucidate the pathways by which ABCA1 regulates glial apoE secretion and lipidation.

Aim 2: To determine whether ABCA1 specifically regulates the secretion of newly synthesized apoE and/or also affects apoE recycling.

Aim 3: To determine the mechanism of action of several synthetic compounds and steroids as apoE modulators for potential therapeutic use, and whether ABCA1 is involved in the process.

The following chapters aim to address these objectives as Chapter 2 presents published results that focus on Aim 1 and Aim 2, while Chapter 3 presents the study for Aim 3 that is recently published. Chapter 4 summarizes conclusions and discusses future directions.

Chapter 2: An ABCA1-independent pathway for recycling of a poorly lipidated 8.1 nm apolipoprotein E particle from glia

2.1 Introduction

High density lipoprotein (HDL) mediates reverse cholesterol transport (RCT), which is the process by which excess cholesterol is collected from peripheral tissues and delivered to the liver for biliary excretion or to steroidogenic organs for steroid hormone biosynthesis (Lund-Katz & Phillips, 2010). The rate-limiting step in HDL biogenesis is catalyzed by the cholesterol transporter ABCA1, which effluxes cholesterol and phospholipids from the plasma membrane to apolipoprotein acceptors (Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Rust et al., 1999). In plasma, the primary apolipoprotein acceptor for ABCA1 is apolipoprotein A-I (apoA-I), whereas in the central nervous system it is apoE (Hirsch-Reinshagen et al., 2004; Wahrle et al., 2004). Deficiency of ABCA1 causes Tangier Disease (TD), which is characterized by a nearly total loss of plasma HDL and increased catabolism of poorly-lipidated apoA-I in the kidney (Fredrickson et al., 1961). TD subjects also present, to various degrees, with hepatosplenomegaly, tissue cholesteryl ester deposition, peripheral neuropathology and a moderate increase in cardiovascular disease (CVD) risk (Fredrickson et al., 1961). Despite the well-known effects of ABCA1 mutations on peripheral lipid and HDL metabolism, there are no reports of the impact of ABCA1 deficiency on brain HDL metabolism in humans.

The brain is the most cholesterol rich organ in the body, containing over 25% of total body cholesterol in only 2% of total body weight (Dietschy & Turley, 2001). Dysfunctional brain lipid

metabolism contributes to several neurodegenerative disorders, including Alzheimer's Disease (AD) (J. Fan et al., 2009), Smith Lemli Opitz syndrome (Chattopadhyay & Paila, 2007) and Neiman-Pick Disease type II (Vanier, 2010). Despite the importance of brain lipid metabolism for lifelong neuronal function, less is known about lipid and lipoprotein metabolism in the central nervous system (CNS) compared to non-CNS tissues, primarily because CNS lipid metabolism is largely segregated from that of plasma. Under normal conditions, dietary cholesterol does not cross the blood-brain-barrier (BBB) and all cholesterol required in the CNS is synthesized in situ (Dietschy & Turley, 2001). Unlike plasma, the CNS does not contain apoB-containing lipoproteins including low-density lipoproteins (LDL), very low-density lipoproteins (VLDL) and chylomicrons (Ladu et al., 2000a). Instead, cholesterol and other lipids in the CNS are transported on lipoprotein particles that resemble plasma HDL in size and density (Ladu et al., 2000a). ApoA-I is present in cerebrospinal fluid (CSF) at approximately 0.5% of its levels in plasma and may enter CSF by transfer across the BBB or via expression in choroid plexus epithelial cells (Demeester et al., 2000; Koch et al., 2001; Pitas et al., 1987b; Saito et al., 1997; Song et al., 1997). In contrast, full-length apoE does not cross the BBB (Linton et al., 1991) and the apoE in the CNS is synthesized and secreted by astrocytes and microglia (Ladu et al., 1998; Lee & Landreth, 2010).

ApoE is a 299 amino acid protein that, in humans, is expressed as three genetic variants that differ by a single amino acid (Mahley & Rall, 2000). These variants are apoE2 (cys112, cys158), apoE3 (cys112, arg158) and apoE4 (arg112, arg158) (Mahley & Rall, 2000). ApoE genotype is the most established genetic risk factor for Alzheimer's Disease (AD) in the general population (Bertram et al., 2010). Carriers of the APOE ϵ 4 allele display increased AD prevalence and an

earlier age of onset in a gene dose-dependent manner (Corder et al., 1993), whereas inheritance of the APOE ϵ 2 allele delays the age of onset and reduces AD prevalence (Corder et al., 1994). Although the precise mechanisms by which apoE contributes to AD pathogenesis is not entirely understood, apoE has a significant influence on the metabolism of A β peptides, which are the toxic species that accumulate as amyloid plaques in the neural parenchyma and cerebrovasculature of AD patients (J. Fan et al., 2009).

We and others have recently shown that the amount of lipids carried by apoE is an important determinant of A β metabolism in mice. Specifically, ABCA1^{-/-} mice have poorly-lipidated apoE that exacerbates the formation of amyloid plaques (Hirsch-Reinshagen et al., 2005; R. Koldamova et al., 2005; Wahrle et al., 2005), whereas selective overexpression of ABCA1 by 6-fold of more results in lipid-enriched apoE that inhibits amyloidogenesis (Wahrle et al., 2008). Recently, ABCA1-mediated lipidation of apoE was shown to facilitate the proteolytic degradation of A β peptides by neprilysin and insulin-degrading enzyme (Q. Jiang et al., 2008). In addition to AD, apoE4 genotype is also associated with a worse prognosis after several types of acute neuronal insults (Verghese et al., 2011). ApoE levels are presumably elevated in the injured brain to scavenge the vast amount of lipids released by degenerating neurons and myelin, which are later delivered to surviving neurons during reinnervation and synaptogenesis. These observations underscore the importance of understanding CNS apoE-HDL metabolism for both acute and chronic neurological applications.

In plasma, HDL is not a single entity but rather exists as a complex and dynamic mixture of subclasses that differ in size as well as cholesterol, lipid and protein composition (Cavigiolio et

al., 2008; Rothblat & Phillips, 2010). These differences endow each HDL subclass with unique physiological properties with respect to receptor affinity, enzyme and protein association, cholesterol efflux capacity, size and stability. Subclass-specific properties are important aspects of RCT, where HDL must first accept lipids, become stabilized for transport through the bloodstream and then offload its lipid cargo at target tissues (Cavigiolio et al., 2008; Rothblat & Phillips, 2010). ApoA-I, the key protein component of plasma HDL, is expressed in liver and intestine and upon synthesis is rapidly lipidated by ABCA1 to form nascent discoidal HDL particles, with liver ABCA1 generating approximately 70% of plasma HDL whereas intestinal ABCA1 contributes the remaining 30% (Brunham et al., 2006; Timmins et al., 2005). These newly synthesized nascent HDL particles have a discoidal shape, are composed of two or three molecules of apoA-I and form several distinct particle subtypes (Lund-Katz & Phillips, 2010). For example, reconstituted nascent apoA-I HDL particles range from 7.8 to 17.0 nm in diameter and differ in their abilities to stimulate ABCA1-dependent cholesterol efflux and to bind and activate lecithin cholesterol acyltransferase (LCAT) (Lund-Katz & Phillips, 2010). The LCAT reaction converts free unesterified cholesterol to cholesteryl ester (CE) during the maturation of immature nascent discoidal particles to mature spherical HDL (Asztalos et al., 2007), which is the preferred substrate for SR-BI-mediated cholesteryl ester (CE) uptake by the liver and steroidogenic organs (Acton et al., 1996).

Compared to nascent apoA-I HDL particles in the periphery, less is known about the structure, function and metabolism of nascent apoE-HDL particles in the brain. Previous studies have shown that nascent apoE and apoJ are secreted as distinct particles from cultured primary astrocytes (DeMattos et al., 2001; A. M. Fagan et al., 1999). After 72 h of media conditioning,

apoJ is found on particles approximately 8-11 nm in diameter with an irregular structure and containing approximately 3 parts protein and one part ethanolamine glycerophospholipids, whereas apoE forms nascent discoidal particles approximately 8, 11 and 14 nm in diameter, composed roughly of two parts protein, one part unesterified cholesterol and one part phospholipids, with ethanolamine glycerophospholipids being approximately 3-fold more abundant than choline phospholipids (DeMattos et al., 2001; A. M. Fagan et al., 1999).

Similar to its role in plasma HDL biogenesis, ABCA1 has critical functions in the generation of apoE HDL in the CNS (Hirsch-Reinshagen & Wellington, 2007). Mice deficient in ABCA1 have an 80% loss of total apoE protein levels in brain tissue and exhibit reduced levels and abnormally small apoE lipoprotein particles in CSF (Hirsch-Reinshagen et al., 2004; Wahrle et al., 2004). Nascent apoE HDL particles secreted by ABCA1^{-/-} glia are smaller and contain approximately 30% of the cholesterol found on WT particles (Wahrle et al., 2004). Intriguingly, ABCA1^{-/-} glia secrete less apoE during the first 6-8 h of media conditioning (Hirsch-Reinshagen et al., 2004), but by 72h total secreted apoE levels become equivalent between WT and ABCA1^{-/-} cells (Wahrle et al., 2004). These observations suggest that glial apoE secretion also involves ABCA1-independent pathways. Here we show that ABCA1 facilitates the rapid secretion and lipidation of nascent apoE from primary glia to produce nascent HDL particles ranging from 7.5-17 nm in diameter. An ABCA1-independent pathway promotes the secretion of a poorly-lipidated 8.1 nm apoE subspecies from both WT and ABCA1^{-/-} glia, which is recycled primarily through the LDL receptor (LDLR).

2.2 Materials and methods

2.2.1 Animals

ABCA1^{-/-} mice were obtained from Dr. Omar Francone (Pfizer Global Research and Development (Groton) and are on a DBA/1LacJ genetic background (McNeish et al., 2000). LDLR^{-/-} and apoE^{-/-} mice, each on a C57Bl/6 background, as well as C57Bl/6 inbred mice, were obtained from Jackson Laboratories (Bar Harbor). Double LDLR/ABCA1^{-/-} mice were generated by intercrossing single knockout lines followed by one backcross. Animals were maintained on a standard chow diet (PMI LabDiet 5010, containing 24% protein, 5.1% fat, and 0.03% cholesterol). All procedures involving experimental animals were performed in accordance with approved protocols from the Canadian Council of Animal Care and the University of British Columbia Committee on Animal Care.

2.2.2 Primary glial culture

Primary mixed glia were prepared from post-natal day 0-2 pups as described (Hirsch-Reinshagen et al., 2004). Brains were removed and placed in ice-cold Hanks Buffered Salt Solution (HBSS) adjusted to 6 g/L glucose and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer. Meninges were removed, cortices and hippocampi were isolated and tissues samples were minced with forceps and then homogenized by gentle trituration through a 5 mL pipette. Suspensions were centrifuged at 1,000 rpm for 4 min. The supernatant was discarded and cells were resuspended in 3 mL of growth media (DMEM:F12 containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% penicillin/streptomycin, Invitrogen). Each preparation from an individual pup was seeded into one T75 tissue culture flask with 13-15 ml growth media. Media was changed every 3-5 days until cells were fully confluent at approximately 14-

20 days. Once confluent, cells were washed once with serum-free conditioning media and then incubated with serum-free conditioning media consisting of 1:1 DMEM:F12 with 0.2 mM penicillin, 0.05 mM streptomycin for 72 h unless otherwise indicated. Glial conditioned media (GCM) was harvested and centrifuged at 1,000 x g for 3 minutes to remove any remaining cells, then concentrated 10 X using Vivaspin 15 centrifugal concentrators with a molecular weight cut-off of 10,000 Daltons (Sartorius Mechatronics). GCM was stored at -20°C until analyzed. For cellular analysis, cells from one T75 flask were collected after incubating with 3 ml of 0.25% trypsin with EDTA for 5 min and centrifugation at 1,000 x g for 3 min. Cell pellets were washed once with PBS. For protein analysis, cell pellets were lysed in 500 µl of RIPA lysis buffer (20 mM Tris, 1% NP40, 5 mM EDTA, 50 mM NaCl, 10 mM Na pyrophosphate, 50 mM NaF, and Complete protease inhibitor, pH 7.4). Cell lysates were sonicated at 30% output for 10 sec. Overall protein concentration was determined by Lowry protein assay (Bio-rad, Hercules, CA). Glial lysate samples were kept at -80 °C until analyzed by 10% SDS-PAGE.

2.2.3 Fluorescent activated cell sorting (FACS)

Mixed glial cells were detached from a T75 culture flask with 0.25% trypsin for no more than 2 min, washed 3 times with ice-cold PBS and triturated to a single cell suspension in FACS buffer (2% FBS in 1 x PBS). Cells were distributed into 96-well U-bottomed plates at approx. 1 x 10⁶ cells per well and manipulated in the dark. Cells were incubated on ice for 30 min with 1:50 PE-anti-mouse CD11b antibody (BD Pharmingen #557397). After two washes with FACS buffer, cells were fixed and permeabilized with BD Cytfix/Cytoperm solution (BD Biosciences #554722) for 30 min on ice, washed twice with saponin-containing BD Perm/Wash buffer (BD Biosciences #554723) and incubated with 1:25 Alexa Fluor 488-conjugated-anti-mouse GFAP

antibody (Cell Signalling #3655) on ice for 30 min. After another two washes with saponin-containing buffer, the cells were resuspended in 2% PFA prior to FACS analyses. Cell fluorescence signals were determined by using a FACS Calibur (Becton Dickinson) equipped with highly sensitive blue (480 nm) and red (635 nm) lasers for quantitative analysis applications. Data was acquired and analyzed using CellQuestPro software (Becton Dickinson).

2.2.4 Conditioned media exchange

72 h conditioned media were collected from a whole T75 flask of wild-type or ABCA1^{-/-} primary glia and centrifuged at 1,000 x g for 3 min to remove cellular debris. Half of the collected media was stored at -20°C and the other half was transferred to a T75 flask of apoE^{-/-} glia for another 48 h incubation. All collected media were concentrated 10X as above prior to analysis.

2.2.5 Electrophoresis and western blotting

For native PAGE, GCM samples were mixed with non-denaturing loading dye to a final concentration of 0.04% bromophenol blue, 4.0% glycerol and 100 mM Tris pH 6.8) and resolved on 6% non-denaturing Tris-HCl polyacrylamide gels. For SDS-PAGE, media and cell samples were mixed with loading dye containing 2% SDS and 1% β-mercaptoethanol, incubated for 10 min at 90°C and resolved on 10% Tris-HCl polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride (PVDF, Millipore) membranes at 24 V overnight at 4°C. After blocking with 5% non-fat milk in PBS for 1 h, membranes were probed with 1:1000 goat-anti murine apoE (Santa Cruz, Chemicon), 1:2500 goat-anti human apoE (Biodesign), 1:1000 rabbit-anti human apoJ (Signet), 1:1000 monoclonal anti-ABCA1 (a gift from Dr. Michael Hayden),

1:1000 goat-anti-LDLR (R&D Systems), 1:2000 rabbit-anti-LRP (a gift from Dr. Joachim Herz) overnight at 4°C. The Biodesign antibody to human apoE cross-reacts with murine apoE. Membranes were washed with 2xPBS-T (2xPBS with 0.05% Tween-20) 4 times for 8 min each and incubated for 1 h with 1:1000 horseradish peroxidase (HRP)-labeled anti-goat secondary antibody (Santa Cruz). Results were visualized using chemiluminescence (ECL, Amersham, New Jersey, USA). Particle diameters on native gels were determined by comparison with a native high molecular weight marker standard (Amersham). Protein molecular weights on denaturing gels were determined by comparison with the Kaleidoscope molecular weight marker standard (BioRad).

2.2.6 Potassium bromide (KBr) gradient ultracentrifugation

Three ml of CGM, conditioned for 72 h and concentrated 5 X, was adjusted to a density of 1.25 g/ml with KBr salt and overlaid with 2.0 ml of 1.225 g/ml, 4.0 ml of 1.100 g/ml and 3.0 ml of 1.006 g/ml KBr solutions. Gradients were centrifuged for 21h 15 min at 40,000 rpm at 20°C in a SW41 rotor (Beckman). Four fractions were collected from the top of the gradient: Fraction 1 = 4 ml (1.060 g/ml), fraction 2 = 3 ml (1.122 g/ml), fraction 3 = 3 ml (1.195 g/ml) and fraction 4 = 2 ml (1.252 g/ml). Fractions were dialysed against PBS at 4°C overnight, then concentrated 10 X using Centriprep YM-10 concentrators with molecular weight cut-off of 10,000 Daltons (Millipore) and analyzed by 6% native PAGE and 10% SDS-PAGE. TC content of fractions was measured using commercially available enzymatic kits (Invitrogen) following the manufacturer's instructions.

2.2.7 Electron microscopy

350 μ L of concentrated GCM was dialyzed against electron microscopy (EM) dialysis buffer (0.125 M ammonium acetate, 2.6 mM ammonium carbonate and 0.26 mM EDTA) overnight at 4°C. 2 μ L of each sample was then mixed with 2 μ L of 2% sodium phosphotungstate and incubated for 1 min. 5 μ L of the stained sample was loaded onto formvar- and carbon-coated copper grids (Canemco). Excess solution was immediately absorbed with filter paper and the sample was air-dried on the grid. Particles were imaged on a Hitachi H7600 transmission electron microscope using an AMT Advantage HR Digital CCD camera.

2.2.8 Oil-Red-O staining

Glial cells were seeded in poly-D-lysine coated 12-well plates at 300,000 cells/well. After 24 h in DMEM containing 10% fetal FBS, cells were air dried, fixed in neutral-buffered formalin and stained with Oil-Red-O. Nuclei were counterstained with hematoxylin. Cells were photographed on a Zeiss Axioplan 2 microscope using a CCD camera equipped with AxioVision Rel. 4.6 (Carl Zeiss Inc.) imaging software.

2.2.9 RAP purification

A single colony of BL21 E. coli containing pGEX-KG-RAP (a gift from Dr. Joachim Herz) was inoculated into 10 mL of 2x YT media and cultured overnight at 37°C, with shaking at 220 rpm. The 10 mL culture was diluted with 1 L of 2x YT media and cultured using the same conditions until OD₆₀₀ = 0.8-1.0. Expression of GST-RAP was induced by adding 0.5 M IPTG to a final concentration of 0.2 mM for 1 h. Cells were harvested by centrifugation at 5,000 rpm for 10 min at 4°C, washed with 1x PBS and centrifuged as before. Cells were resuspended in 60 ml of lysis

buffer (1x PBS, protease inhibitor, 10% glycerol and 1% Triton X-100 and fresh lysozyme powder [100mg/mL]) and incubated on ice for 15 min. Cells were sonicated three times for 15 sec at 20% amplitude and cleared by centrifugation at 12,000 rpm for 15 min. The supernatant was then incubated for 3 h at room temperature (RT) with 1 mL glutathione sepharose beads (previously equilibrated with 1x PBS) for every liter of culture. Beads were centrifuged at 2000 rpm and washed 3 times with 1x PBS. Beads were then equilibrated with 1x precision protease buffer (50 mM Tris-Cl pH 7.0, 150 mM NaCl, 1 mM EDTA and 1 mM DTT) before incubation with protease solution (30 units in 1 mL of protease buffer per 1 L culture) at RT overnight. Eluate was collected, and another 1 mL of fresh protease buffer was added to the beads and incubated at RT overnight. Eluate samples were pooled and concentrated 10 X using Centriprep YM-10 concentrators (Millipore) and analysed on a 10% SDS-PAGE gel stained with Coomassie Blue for quantification.

2.2.10 RAP inhibition of apoE uptake

Wild-type and ABCA1^{-/-} glia were reseeded into 12-well plates at a density of 400,000-500,000 cells/well in 1 mL of maintenance media (DMEM:F12 containing 10% FBS, 2 mM L-glutamine, 1% penicillin/streptomycin). After 48 h, cells were treated with either 500 μ L of serum-free conditioning media (DMEM:F12) or serum-free conditioning media containing 1 μ M BSA, 70 nM RAP or 1 μ M RAP for 72 h. Conditioned media was collected and cells were harvested using 100 μ L of RIPA lysis buffer followed by Western blot analysis on non-denaturing or denaturing gels as described above.

2.2.11 ApoE ELISA

96 well ELISA plates (Fisher, costar cat. #3590) were coated with 3.13 ng/well WUE4 apoE antibody at 4 °C overnight, then washed five times with PBS-0.025% Tween (pH 7.4), blocked with 1% non-fat dry milk in PBS (pH 7.4) for 1 h and washed as above. Standards or samples were diluted with Dilution Buffer (DB, 0.5% BSA, 0.025% Tween 20 in PBS, pH 7.4) and heated at 52 °C for 3 hr. Wells were loaded with 100 µl of sample in duplicate and 100 µl of apoE standards in triplicate, incubated in a humid chamber for 24 h at 4 °C, then aspirated and washed as before. 100 µl per well of Calbiochem anti-apoE (1:12,000) in DB was then added and incubated for 90 min at 37 °C. After washing, 100 µl per well of biotinylated anti-goat IgG (1:160,000, Vector) in DB was added and incubated for 90 min at 37 °C. Streptavidin-poly-HRP antibody (1:4000, Pierce) was then added and incubated at RT for 90 min. Color was developed by adding 100 µl/well Sigma ELISA TMB and stopped immediately by adding 100 µL of 1M HCL. Plates were read at 450nm absorbance.

2.2.12 Cholesterol and phospholipid measurement

Total cholesterol measurements of GCM samples were performed using the fluorogenic Amplex Red Cholesterol Assay Kit (Invitrogen) following the manufacture's protocol. To measure total cholesterol and phospholipids levels in cells, cellular lipids were extracted using a modified Bligh and Dyer method. Briefly, a cell suspension was added to 1:2 (v/v) chloroform:methanol, vortexed for 30 sec and centrifuged at 1,000 x g for 5 min. The supernatant was then transferred to 1:1 (v/v) chloroform:0.9% NaCl solution. After another centrifugation at 1,000 x g for 5 min, the chloroform phase was transferred to new glass tube and dried under nitrogen. After the lipid pellet was dissolved in 2% Triton-X 100, cellular total cholesterol and phospholipids content

were measured by Wako kits following the manufacture's protocols (Wako #439-35801 for Cholesterol E kit, #433-36201 for Phospholipids C kit).

2.2.13 Preparation of recombinant apoA-I

Human apoA-I was expressed in bacteria as previously described (Ryan et al., 2003). The protein was expressed in *Escherichia coli* strain BL21 (DE-3) pLysS and cultured in NZCYM media containing 50 µg/ml ampicillin. Expressed proteins were purified via Hi-Trap nickel chelating columns (GE Biosciences, Inc., Piscataway, NJ) as described (Ryan et al., 2003). Protein purity (>95%) was confirmed by SDS-PAGE.

2.2.14 Statistical analysis

Data are shown as mean ± standard error. One-way ANOVA with a Tukey's Multiple Comparison post test or two-tailed unpaired Student's t-tests were used for statistical analysis. For t-test analyses, Welch's correction for unequal variances was applied when variances were significantly different between groups. All statistical analyses were performed using GraphPad Prism (version 5.0; GraphPad Software for Science Inc., San Diego, CA).

2.3 Results

2.3.1 Nascent apoE particle subspecies are secreted from WT and ABCA1^{-/-} glia in a distinct temporal pattern

Previous studies have suggested that ABCA1^{-/-} glia secrete less apoE within the first 6-8 h of conditioning compared to WT glia but that total apoE levels become equivalent after 72 h (Hirsch-Reinshagen et al., 2004). To further delineate the dynamic nature of glial apoE secretion, we characterized the electrophoretic pattern of apoE subspecies secreted from mixed primary WT and ABCA1^{-/-} glia after 6, 24, 48, and 72 h of conditioning. FACS analysis shows that WT and ABCA1^{-/-} cultures contain comparable amounts of GFAP-positive astrocytes and CD11b-positive microglia (astrocytes: WT= 80.71 ± 4.73 %, N=5, ABCA1= 84.07 ± 3.39 %, N=5, $p > 0.05$; Microglia: WT= 2.68 ± 1.17 %, N=5, ABCA1= 3.25 ± 1.95 %, N=5, $p > 0.05$). Native PAGE analysis shows that WT glia secrete several apoE nascent particles ranging in diameter from approximately 7.5 – 17 nm, including an 8.1 nm species that requires at least 48 h of conditioning to become detectable in WT glial conditioned media (GCM) (**Fig. 2.1**). ABCA1^{-/-} glia secrete only the 8.1 nm particle. SDS page analysis confirms that ABCA1^{-/-} glia secrete less total apoE during the first 6 h of conditioning.

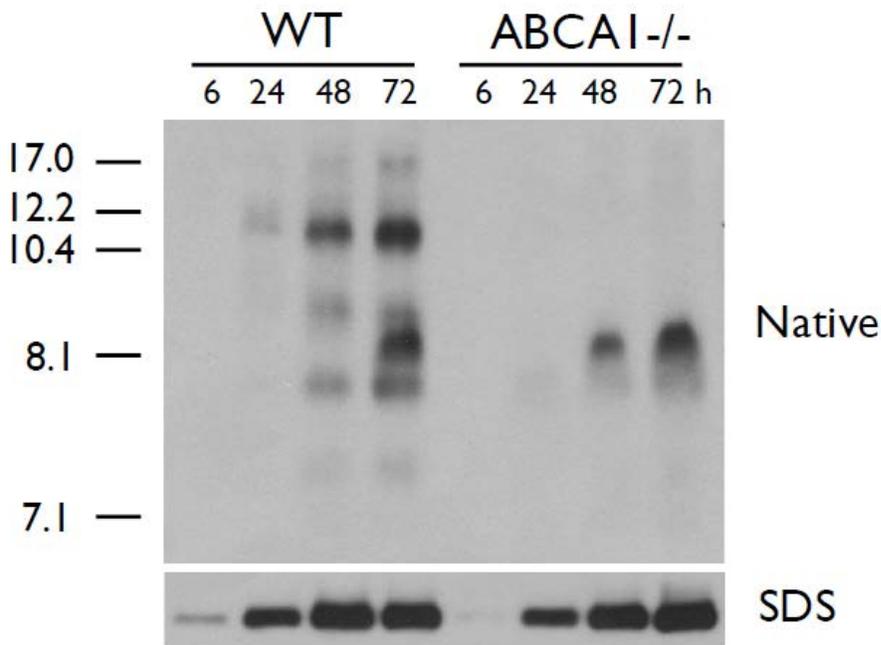


Figure 2.1: Glial apoE is secreted as distinct nascent subspecies.

Wild-type (WT) and ABCA1 deficient (ABCA1^{-/-}) primary mixed glia (97-98% astrocytes, 2-3% microglia) were cultured to confluency and then conditioned in serum-free media for indicated time points. Media was collected, concentrated 10-fold and analysed by 6% native PAGE followed by immunoblotting for apoE. Stokes diameter of standards are listed on the left.

2.3.2 The 8.1 nm particle secreted from WT and ABCA1^{-/-} glia is a lipid poor and structurally distinct species

Wahrle et al. previously reported that the total cholesterol (TC) content of conditioned media from ABCA1^{-/-} astrocytes is reduced by 70% compared to WT controls (Wahrle et al., 2004), indicating that the 8.1 nm species is lipid poor. To confirm this observation, we quantified the TC and total apoE levels in 72h GCM by enzymatic assay and ELISA, respectively, and observed a 79% decrease in TC (WT: 72.3 $\mu\text{g TC}/\mu\text{g apoE}$; ABCA1^{-/-}: 15.1 $\mu\text{g TC}/\mu\text{g apoE}$, $p < 0.001$), consistent with Wahrle's earlier observation (Wahrle et al., 2004). To determine if there were differences in lipidation of individual glial-derived nascent apoE subspecies, we next performed KBr gradient ultracentrifugation to separate the particles by buoyant density. Western blot analysis of KBr fractions showed that all particles secreted by WT glia except the 8.1 nm species were recovered in fraction 2 (1.1-1.2 g/ml) (**Fig. 2.2A**). In contrast, the 8.1 nm particle from both WT and ABCA1^{-/-} glia was recovered in fraction 4 (> 1.25 g/ml) (**Fig. 2.2A**). ApoJ, a protein-dense lipoprotein secreted from glia, is also recovered in fraction 4 from both WT and ABCA1^{-/-} media (**Fig. 2.2A**). These observations indicate that the 8.1 nm particle, detectable in WT GCM by 48-72 h, is equivalent to the 8.1 poorly lipidated species that is the sole apoE particle found in ABCA1^{-/-} GCM. Negative staining EM of conditioned media from WT and ABCA1^{-/-} glia revealed that rouleaux characteristic of discoidal HDL particles was observed in WT but not ABCA1^{-/-} samples, suggesting that the 8.1 nm particle is nondiscoidal and structurally incapable of forming these distinctive stacks (**Fig. 2.2B**).

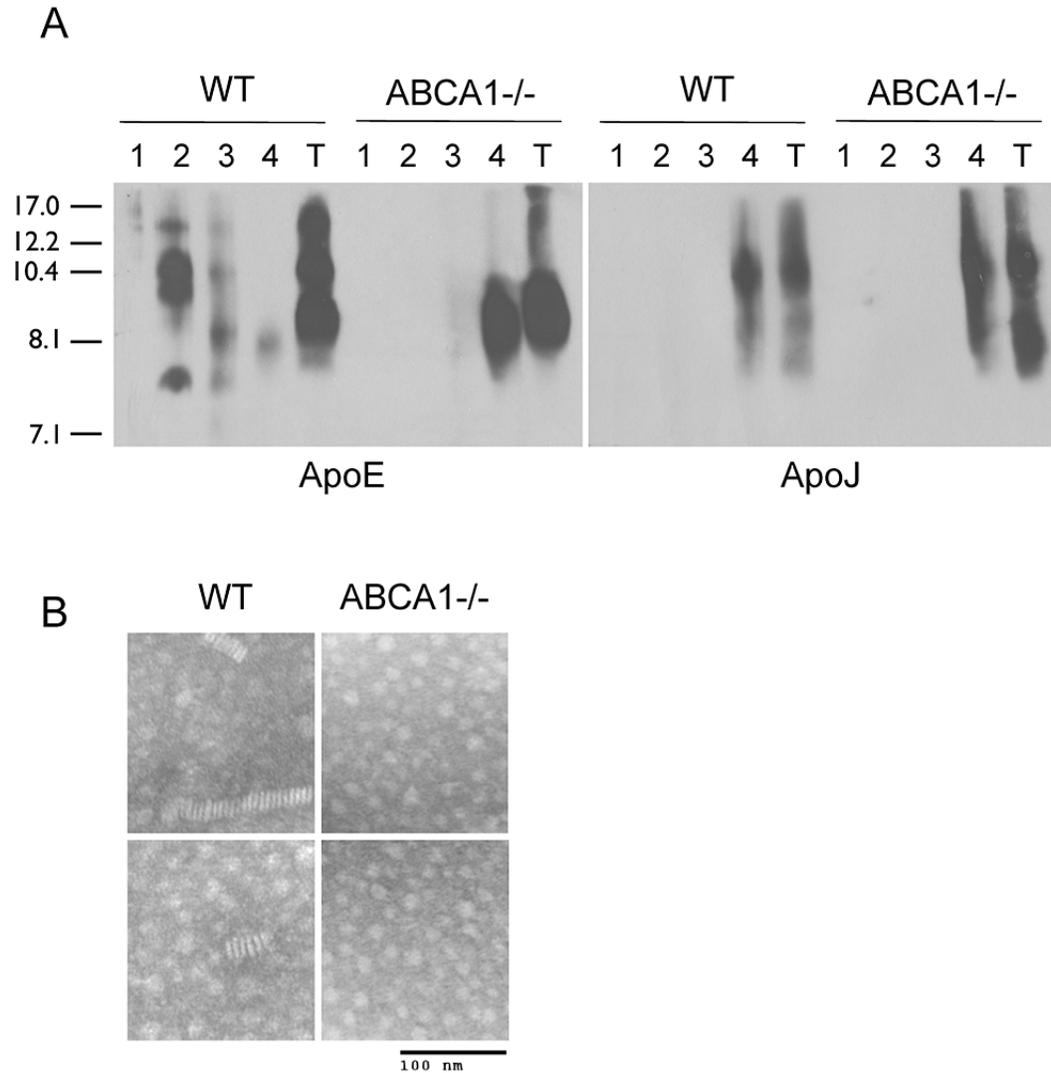


Figure 2.2: The 8.1 nm apoE particle is protein dense and incapable of forming rouleaux. (A) Confluent wild-type (WT) and ABCA1-deficient (ABCA1^{-/-}) primary mixed glia were conditioned in serum-free media for 72h. Media was collected, concentrated 10-fold, and analyzed by KBr ultracentrifugation. Samples were separated into four fractions: Fraction 1 = 1.060 g/ml, fraction 2 = 1.122 g/ml, fraction 3 = 1.195 g/ml, and fraction 4 = 1.252 g/ml. Fractions were dialyzed, concentrated, and analyzed by 6% native PAGE followed by immunoblotting to apoE and apoJ relative to unfractionated total (T) conditioned media. Stokes diameter of standards are listed on the left. (B) Negative stain EM of media conditioned from WT and ABCA1^{-/-} primary mixed glia for 72h, showing two representative images for each genotype and magnification (bar).

2.3.3 Lipidation of the 8.1 nm apoE particle by ABCA1 restores apoE subspecies distribution to resemble the WT pattern

Because the 8.1 nm apoE particle is a structurally distinct lipid-poor HDL particle relative to the other nascent apoE HDL particles secreted by WT glia, we next queried whether it was capable of accepting lipids from ABCA1. Media from WT and ABCA1^{-/-} glia were conditioned for 72 h and then added to apoE^{-/-} glia for an additional 48 h to allow the donor particles to be lipidated by ABCA1 expressed in the recipient cells. Donor apoE particles secreted from both WT and ABCA1^{-/-} glia gave rise to a nearly identical pattern of apoE particles after incubation with ABCA1-expressing apoE^{-/-} glia (**Fig. 2.3**). The only particle that ABCA1^{-/-} donor media failed to generate was a large species at approximately 17 nm. These observations demonstrate that despite its structurally distinct nature, the 8.1 nm apoE particles are not impaired in their ability to accept lipids from ABCA1.

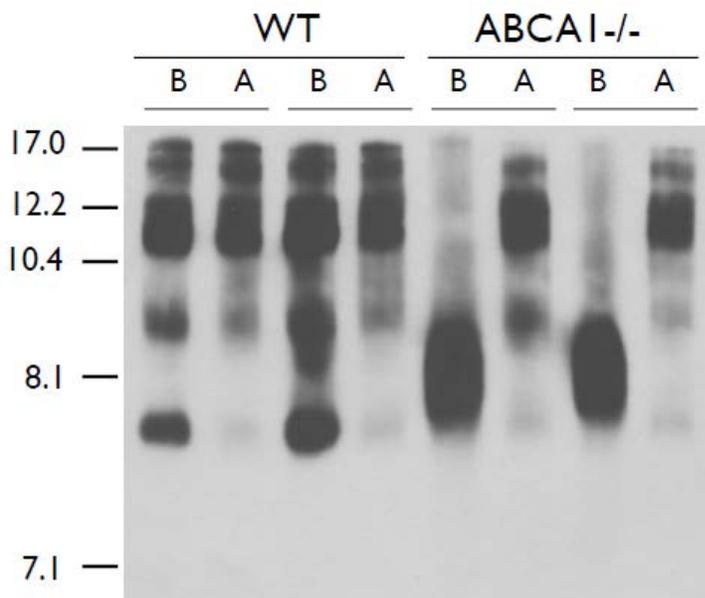


Figure 2.3: The 8.1 nm apoE particle is an efficient lipid acceptor for ABCA1.

Confluent wild-type (WT) and ABCA1-deficient (ABCA1^{-/-}) primary mixed glia were conditioned in serum-free media for 72h. Media was collected and added to confluent primary apoE^{-/-} mixed glia for an additional 48h, then analysed by 6% native PAGE followed by immunoblotting to apoE. The gel shows media samples from two independent animals per genotype before (B) and after (A) exposure to apoE^{-/-} glia. Stokes diameter of standards are listed on the left.

2.3.4 ABCA1 deficiency reduces LDLR levels in cultured glia

We have previously shown that ABCA1-deficient glia accumulate lipids under standard culture conditions (Hirsch-Reinshagen et al., 2004). We confirmed and extended these observations by first staining for neutral lipids using Oil-Red-O in WT, ABCA1^{-/-} and LDLR^{-/-} glia. As expected, we observed minimal lipid droplets in WT glia, abundant lipid droplets in ABCA1^{-/-} glia and no lipid accumulation in LDLR^{-/-} glia (**Fig. 2.4A**). Because cellular lipid accumulation in ABCA1^{-/-} cells is expected to activate SREBP2 that leads to negative feedback regulation of LDLR but not LRP expression, we next quantified LDLR and LRP levels by Western blot. As expected from the presence of lipid accumulation, ABCA1^{-/-} cells exhibited a 75% reduction in LDLR protein levels compared to WT glia ($p < 0.0001$, **Fig. 2.4B**) but no significant change in LRP levels compared to WT glia. The selective reduction in LDLR levels in ABCA1^{-/-} glia indicates that the ratio of LDLR:LRP is likely lower in ABCA1^{-/-} relative to WT glia under the culture conditions employed. There were no significant differences in cellular apoE levels among WT, ABCA1^{-/-} and LDLR^{-/-} glia (**Fig. 2.4B**).

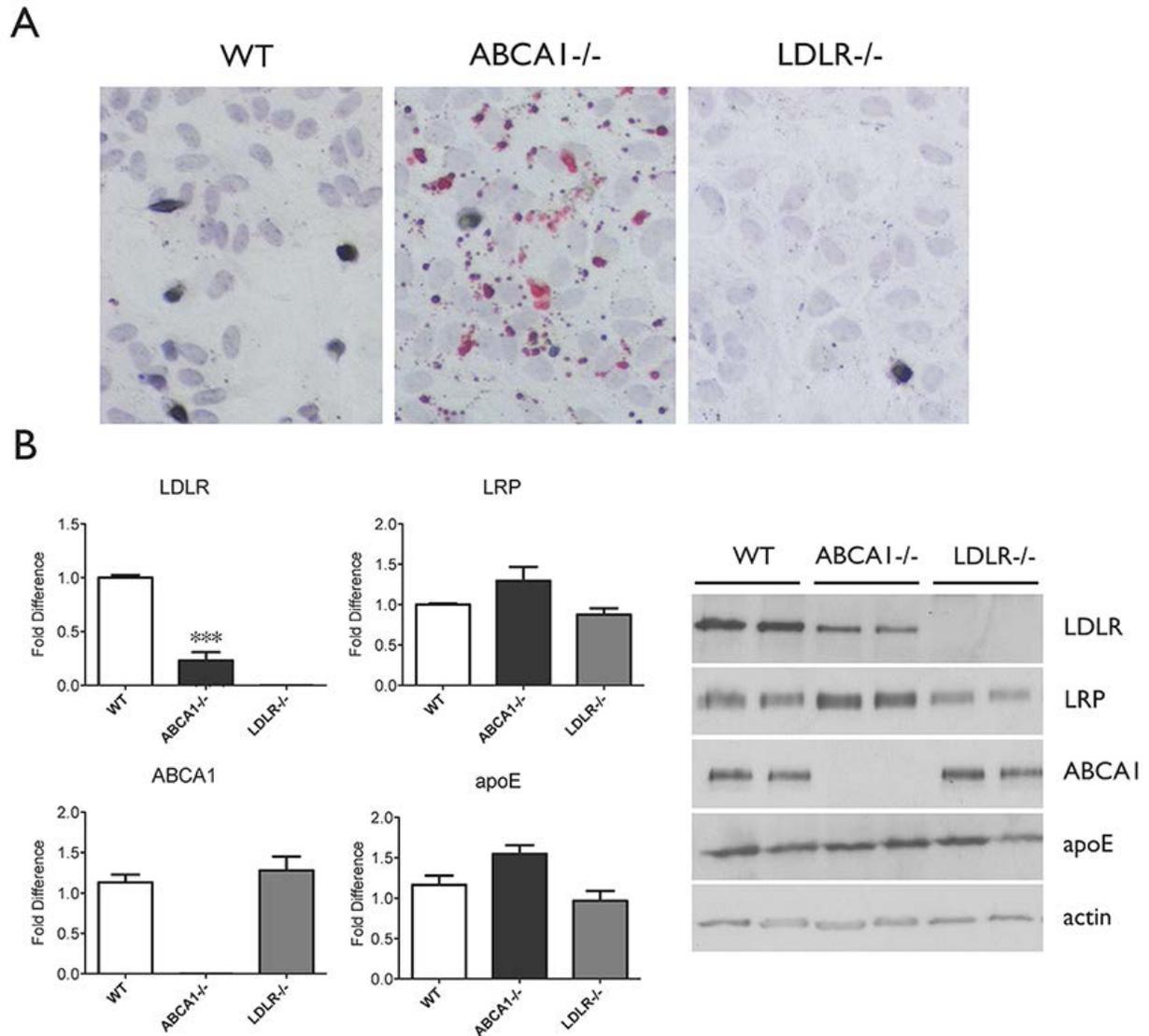


Figure 2.4: ABCA1^{-/-} glia accumulate lipids and have reduced LDLR levels.

(A) Oil-Red-O staining of neutral lipids in WT, ABCA1^{-/-} and LDLR^{-/-} primary mixed glia at confluency. (B) Quantification of cellular LDLR and LRP expression in WT, ABCA1^{-/-} and LDLR^{-/-} primary mixed glia by Western blot. Actin was used as a loading control. Data represent means and standard error of N=6-9 independent cultures, and *** represents $p < 0.001$ using one-way ANOVA with a Tukey's Multiple Comparison Post Test.

2.3.5 Inhibition of apoE receptor activity preferentially reduces secretion of small apoE particles from glia

In several cell types, apoE is recycled through apoE-receptor mediated endocytosis and resecretion (S. Fazio et al., 1999; S. Fazio et al., 2000; Rensen et al., 2000; Swift et al., 2001). To determine if apoE recycling also occurs in primary glia, we first examined the distribution pattern of secreted apoE particles over time in WT and LDLR^{-/-} glia. Appearance of apoE particles in LDLR^{-/-} GCM was delayed relative to WT GCM (**Fig. 2.5**). Furthermore, the 8.1 nm apoE particle was barely detectable even after 96h of conditioning (**Fig. 2.5**). Selective deletion of the LDLR did not alter cellular TC content in LDLR^{-/-} compared to WT glia (WT = 27.09 ± 2.232 ng TC/ng protein, N=4, LDLR^{-/-} = 25.78 ± 1.111 ng TC/ng protein, N=5, p=0.592). These observations are consistent with a role for LDLR in apoE recycling and that the 8.1 nm particle is particularly affected by loss of LDLR activity.

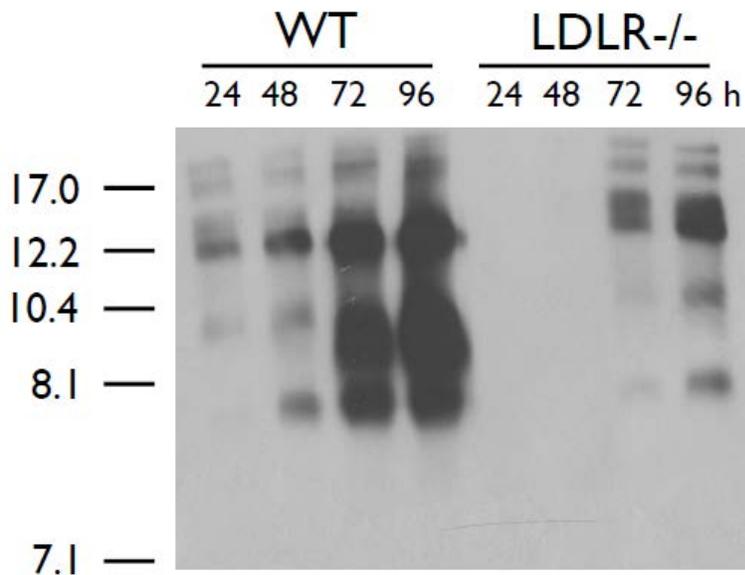


Figure 2.5: LDLR-deficiency leads to a selective loss in the 8.1 nm apoE particle. Wild-type (WT) and LDLR deficient (LDLR^{-/-}) primary mixed glia were cultured to confluency and then conditioned in serum-free media for indicated time points. Media was collected, concentrated 10-fold and analysed by 6% native PAGE followed by immunoblotting for apoE. Stokes diameter of standards are listed on the left.

To further differentiate the roles of glial LRP and LDLR on secretion of apoE subspecies, we also used recombinant RAP, prepared as a GST-fusion protein, to block the activity of apoE receptors. RAP inhibits LRP at 70 nM and both LRP and LDLR at 1 μ M (Ladu et al., 2000b). The levels and pattern of secreted apoE subspecies were not altered when WT, ABCA1^{-/-} and LDLR^{-/-} glia were incubated with 1 μ M BSA as a negative control or with 70 nM RAP, suggesting that LRP does not play a major role in regulating the levels of secreted apoE (**Fig. 2.6A**, left panel). In contrast, treatment of WT and LDLR^{-/-} glia with 1 μ M RAP reduced the total level of secreted apoE, as well as preferentially inhibited the secretion of the smaller apoE subspecies, leaving only the larger 12-17 nm particles evident in GCM (**Fig. 2.6A**, left panel). Although treatment of ABCA1^{-/-} glia with 70 nM RAP had no impact on secreted apoE levels, treatment with 1 μ M RAP blocked the secretion of the 8.1 nm apoE species, virtually eliminating the secretion of apoE (**Fig. 2.6A**, left and right panels). That functional inhibition of apoE receptors reduces secreted apoE is consistent with the hypothesis that apoE recycling, primarily via LDLR, contributes to the steady state apoE levels in GCM. Furthermore, by inhibiting apoE uptake, RAP-mediate inhibition of glial apoE receptors may prolong the extracellular half-life of secreted apoE and increase its availability to ABCA1, thus generating larger particles in cells that are capable of cholesterol efflux.

To specifically delineate the contribution of LDLR to apoE recycling in glia, we generated double ABCA1/LDLR^{-/-} mice and evaluated apoE secretion from primary mixed glia. Densitometric analysis of apoE levels in GCM by SDS-PAGE suggests at least a 70% reduction in total apoE levels in 72 h DKO GCM (WT: 7.33 ± 4.17 optical units, ABCA1^{-/-}: 7.21 ± 4.23 optical units, DKO: 2.12 ± 1.01 optical units) (**Fig. 2.6B**). More accurate quantitation of total

apoE levels by ELISA shows that apoE secretion is reduced by approximately 93% in 72 h DKO GCM (WT: 123.5 ± 1.66 ng/ml, ABCA1^{-/-}: 123.9 ± 10.83 ng/ml, DKO: 8.72 ± 1.13 ng/ml) (**Fig. 2.6C**). Because LDLR levels in ABCA1^{-/-} glia are only approximately 25% of WT levels under the culture conditions employed here (**Fig. 2.6B, C**), these observations clearly define a major role for the LDLR in ABCA1-independent apoE recycling/secretion, as LRP cannot compensate efficiently for LDLR with respect to apoE recycling.

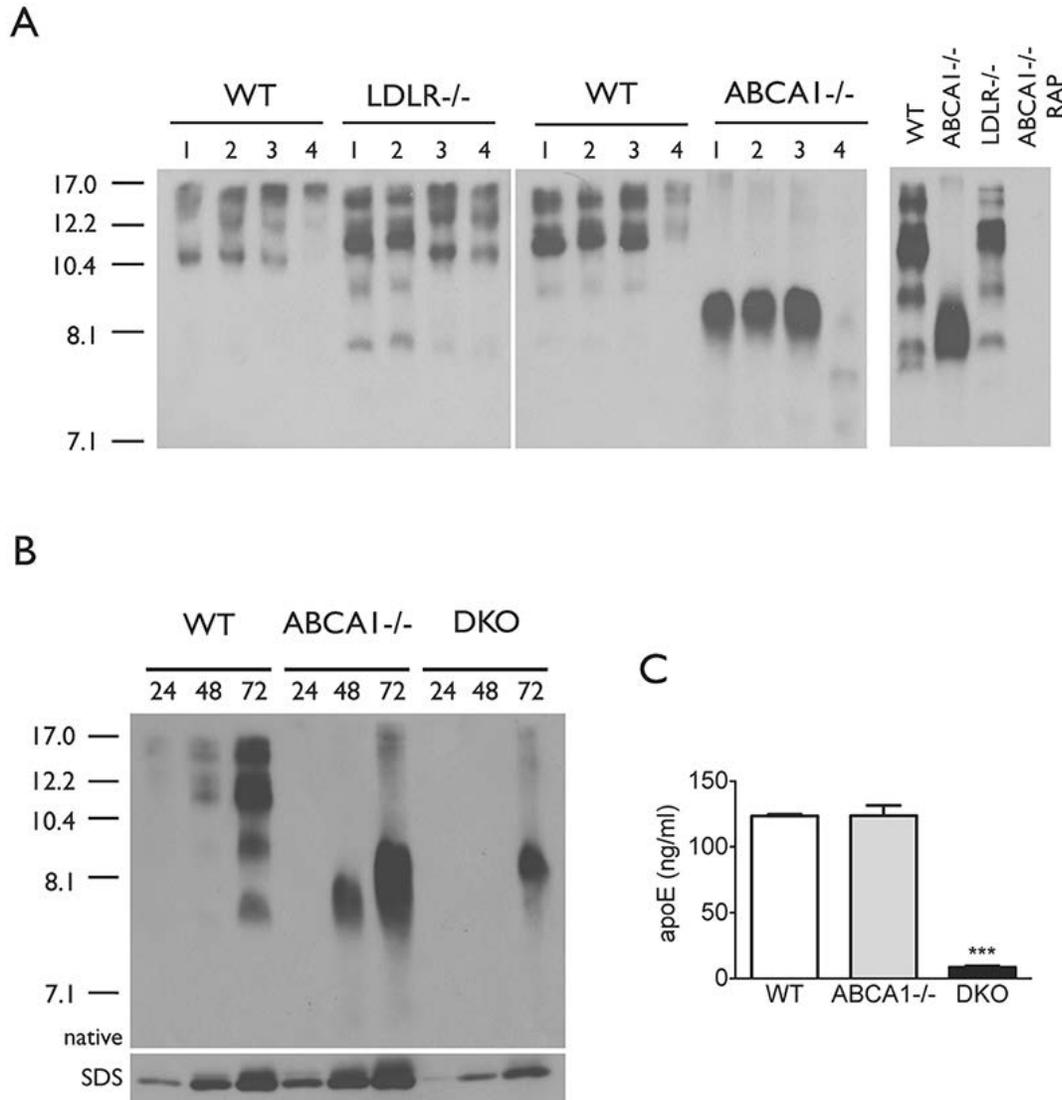


Figure 2.6: Secretion of the 8.1 nm apoE particle involves LDLR function.

(A) Confluent WT, LDLR^{-/-} and ABCA1^{-/-} primary mixed glia were treated with serum-free media containing no additives (1), 1 μ M BSA (2), 70 nM RAP (3) or 1 μ M RAP (4) for 72h (left panel). Confluent WT and LDLR^{-/-} primary mixed glia were conditioned for 72h in the absence of RAP, and primary ABCA1^{-/-} glia were conditioned for 72 with and without 1 μ M RAP as indicated (right panel). Media were collected and analyzed by native PAGE followed by immunoblotting to apoE. (B) Confluent WT, ABCA1^{-/-} and ABCA1/LDLR^{-/-} primary mixed glia were conditioned in serum-free media for 72h. Media was collected at the indicated time points and analyzed by native and SDS PAGE followed by immunoblotting to apoE. Stokes diameter of standards are listed on the left. (C) Quantitation of apoE levels in media from WT, ABCA1^{-/-} and ABCA1/LDLR^{-/-} conditioned media by ELISA. Data represent means and standard error from two independent cultures per genotype using one-way ANOVA with a Tukey's Multiple Comparison Post Test.

2.3.6 ApoA-I stimulates glial apoE recycling and lipidation in primary glia

ApoA-I and HDL have been reported to stimulate apoE recycling in a wide variety of cell types including Chinese hamster ovary (CHO) cells, fibroblasts, hepatocytes, macrophages, adipocytes and neuronal cells (Bencharif et al., 2010; Braun et al., 2006; Dory, 1991; Farkas et al., 2003; Hasty et al., 2005; Heeren et al., 2006; Heeren et al., 2003; Rellin et al., 2008; Rensen et al., 2000). In macrophages, apoA-I-mediated stimulation of apoE secretion is independent of ABCA1 (Kockx et al., 2004). To determine whether apoA-I stimulates apoE secretion from primary glia, we treated mixed glial cultures with recombinant human apoA-I at doses ranging from 10-60 µg/ml for 48 h. Conditioned media showed a clear dose-dependent increase in 8.1nm apoE particles (**Fig. 2.7A**), supporting our hypothesis that this species represents recycled apoE that, in WT cells, has not yet been lipidated by ABCA1. The decreased signal of larger particles (>10nm) in WT media suggested that secreted apoE might be driven more towards the recycling pathway rather than lipidation in the presence of apoA-I. ApoA-I treatment of WT glia also led to a significant increase in cellular LDLR levels, further supporting a role for LDLR in glial apoE recycling (**Fig. 2.7B, C**). It also led to trends toward increased ABCA1 and decreased cellular apoE levels (**Fig. 2.7B, C**). Cellular LRP levels were not significantly affected by apoA-I treatment (**Fig. 2.7B, C**).

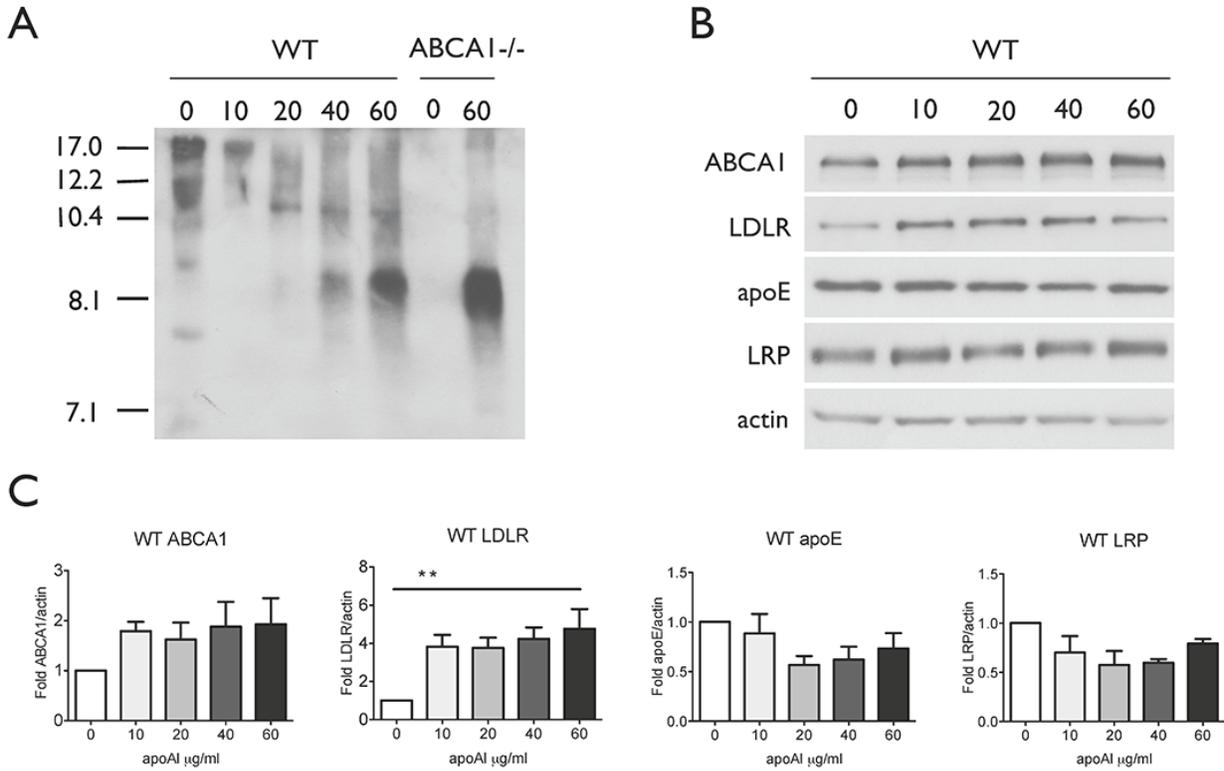


Figure 2.7: ApoA-I stimulates apoE recycling from glia.

(A) WT primary glia were conditioned for 48h in the absence or presence of the indicated concentrations of recombinant human apoA-I. Samples were concentrated 10X and analysed by non-denaturing PAGE and immunodetection for apoE. (B) Cell lysates of primary glia treated with the indicated concentrations of recombinant human apoA-I were separated by SDS-PAGE and immunodetected for ABCA1, LDLR, apoE, LRP and actin as a loading control. (C) Quantitation of cellular ABCA1, LDLR, apoE and LRP levels in WT glia treated apoA-I. Data represent means and standard error from three independent experiments using one-way ANOVA with a Tukey's Multiple Comparison Post Test.

2.4 Discussion

ApoE is at the hub of CNS HDL metabolism and the most important genetic risk factor for sporadic AD. Several lines of evidence suggest that aberrant CNS lipid metabolism may contribute to AD pathogenesis (J. Fan et al., 2009). Of particular interest is the discovery that ABCA1-mediated lipidation of apoE facilitates the degradation and clearance of A β peptides, potentially offering a mechanism for novel therapeutic strategies for AD. Developing such therapeutic approaches will likely require a deeper understanding of the metabolism of brain HDL particles than presently exists. It is likely that CNS apoE-HDL comprise distinct specialized lipoprotein subspecies, similar to complexity of plasma HDL, which are a heterogeneous mixture of subclasses with both structural and functional differences (Cavigiolo et al., 2008; Lund-Katz & Phillips, 2010). In this paper we provide evidence for structural and physiological differences in nascent apoE-derived lipoproteins secreted from glia.

ApoE appears in glial-conditioned media as a variety of nascent particles ranging from 7.5 - 17 nm in diameter, a larger size range than previously reported (DeMattos et al., 2001; A. M. Fagan et al., 1999; Ladu et al., 1998; Wahrle et al., 2004). Most of the earlier studies have allowed media to condition for extended periods of time (i.e. 72h) to allow apoE to accumulate to sufficient levels for detection. We analyzed secreted apoE subspecies from 6-72 h and demonstrate that particle diameters are modulated over this time course, suggesting that there may be extensive remodeling of apoE species that could occur through direct interaction among secreted particles in the media, through interactions at the cellular membrane, or via apoE uptake and recycling mediated by apoE receptors.

In particular, both WT and ABCA1^{-/-} glia secrete an 8.1 nm apoE particle that is structurally and functionally distinct from the other species secreted from WT cells. We hypothesize that this particle is recycled apoE that emerges from the cell in a lipid-poor state independent of ABCA1 and rapidly lipidated if cells express functional ABCA1. Supporting this hypothesis are the following observations. First, the 8.1 nm particle is lipid poor. The 8.1 nm particle is the sole species secreted from ABCA1^{-/-} glia, and previous studies using gel filtration chromatography and media TC measurements have demonstrated its paucity of lipids (Wahrle et al., 2004). Here we show that this 8.1 nm particle is also detectable in WT GCM and that it resolves in a lipid-poor fraction by KBr density gradient ultracentrifugation when isolated from either WT or ABCA1^{-/-} glia. Second, it is structurally distinct from the other apoE species. Negative staining EM indicates that it is unable to form rouleaux characteristic of discoidal lipoprotein particles, consistent with the “figure-8” structure for similarly sized peripheral apoA-I – containing HDL particles predicted by Catta et al. (2006) and observed by Cavigliolo et al. (2008) and Zhang et al. (2011). Nevertheless, it is not a dead-end particle with respect to RCT, as conditioned media swap experiments demonstrate that it is fully capable of accepting lipids effluxed by ABCA1 to form the full repertoire of particles normally observed in WT GCM. Third, blocking apoE receptor function by RAP inhibits the appearance of the 8.1 nm particle from ABCA1^{-/-} glia, suggesting that it may arise through an ABCA1-independent recycling pathway dependent on apoE receptors.

Our data suggests that apoE may be recycled primarily through LDLR in glial cultures. In ABCA1^{-/-} glia, accumulation of lipid droplets triggers feedback regulation that suppresses LDLR expression to approximately 25% of normal levels, but does not significantly impair apoE

secretion after extended conditioning periods. However, decreasing LDLR levels from 25% to zero by selectively deleting LDLR in ABCA1^{-/-} glia greatly impedes apoE secretion to approximately 10% of WT levels. Further, RAP added to levels that would reduce only LRP function did not have an effect on apoE secretion from WT or ABCA1^{-/-} glia, but addition of 1 μ M RAP to inhibit both LRP and LDLR markedly impaired apoE secretion from ABCA1^{-/-} cells. These findings suggest that even though LRP is abundantly expressed in ABCA1^{-/-} glia, it does not compensate well for LDLR in glial apoE recycling.

Intriguingly, lipid-poor apoE particles secreted from human embryonic kidney (HEK) cells were found to self-assemble into a high MW mass that bound more avidly to LRP than LDLR (Ladu et al., 2006). This may be due to the size of these high MW species, as small apoE-containing triglyceride-rich emulsions have impaired LDLR-dependent clearance in rats but emulsions of large particles are cleared independently of LDLR (Rensen et al., 1997). Experiments in primary hepatocytes showed that apoE reconstituted into VLDL particles is recycled normally in LDLR^{-/-} cells even though uptake is reduced by 50% (Farkas et al., 2004). Similar experiments using RAP^{-/-} hepatocytes, which have a 75% reduction in LRP levels, also indicated apoE is recycled normally (Heeren et al., 2001). These results suggest that in hepatocytes, efficient apoE recycling can be mediated by either LDLR or LRP, at least when apoE is reconstituted in VLDL particles. Whether recycling of small nascent apoE-only particles may have a preference for LDLR or LRP in hepatocytes is not yet known. Understanding the differences between high MW HEK-derived apoE particles, reconstituted apoE-VLDL particles and nascent glial-derived apoE particles may offer insight into the factors that govern apoE receptor preference.

Taken together, these results define two pathways that regulate the steady state levels of glial-derived secreted apoE. One pathway requires ABCA1 and leads to the accumulation of several lipidated discoidal nascent apoE particles from 7-17 nm in diameter. The other pathway requires functional apoE receptors, primarily LDLR, and selectively regulates the secretion of a distinct 8.1 nm poorly lipidated apoE species, which is likely re-secreted following LDLR-mediated apoE uptake. Secretion of this particle does not require ABCA1, but when ABCA1 is present the particle is rapidly lipidated and difficult to detect in WT GCM.

Previous studies suggest that 60-80% of internalized apoE may be recycled (S. Fazio et al., 1999; S. Fazio et al., 2000; Rensen et al., 2000; Swift et al., 2001) and directed to HDL particles (Heeren et al., 2001) in non-CNS tissues. For example, triglyceride-rich lipoproteins taken up by hepatocytes via apoE receptors are differentially sorted after endocytosis. Core lipids and apoB are targeted to lysosomes whereas the majority of apoE is delivered to recycling endosomes where it can be mobilized by apoA-I or HDL to be recycled back to the plasma membrane followed by resecretion and relipidation of apoE to form apoE-HDL (Heeren et al., 2003; Rensen et al., 2000). In macrophages, FPLC analysis demonstrates that recycled apoE is present on small HDL, suggesting that it either exits the cell in a lipidated form or is swiftly lipidated by ABCA1 (Hasty et al., 2005). The results presented in this paper suggest that glia also share these pathways.

HDL or purified apoA-I stimulates the release of internalized apoE from CHO cells, hepatocytes, fibroblasts, adipocytes, macrophages and neuronal cells. The mechanism by which apoA-I or HDL stimulates apoE recycling is not yet clear. In hepatoma cells, HDL-derived apoA-I

colocalizes with apoE in EEA-1 positive early endosomes (Heeren et al., 2003). ABCA1 colocalizes with internalized apoA-I in macrophages (Takahashi & Smith, 1999) and modulates late endocytic trafficking (Neufeld et al., 2004). These observations suggest that ABCA1, apoA-I and apoE may have intracellular interactions that promote apoE recycling. Much remains to be learned, however, as internalized apoA-I is apparently not resecreted efficiently and has been reported to contribute to only 1.4% of total HDL production (Denis et al., 2008). Further, because secreted apoE levels are equivalent between WT and ABCA1^{-/-} glia after extended conditioning times (Wahrle et al., 2004), ABCA1 is clearly not required for apoE recycling in glia. These observations parallel the previous observation that apoA-I stimulates apoE secretion from macrophages independent of ABCA1 (Kockx et al., 2004).

Because most of the apoA-I in the CNS is found in CSF and is not synthesized by glia (Demeester et al., 2000; Koch et al., 2001), the relevance of apoA-I-mediated stimulation of apoE recycling in the CNS is not immediately evident. However, several recent studies suggest that apoA-I affects CNS function and impacts the pathogenesis of AD. For example, although deletion of apoA-I alone has no effect on memory, apoA-I^{-/-} mice crossed with the APP/PS1 mouse model of AD exhibit increased inflammation and impaired spatial learning and memory retention compared to APP/PS1 controls (Lefterov et al., 2010). Conversely, two-fold over-expression of apoA-I protected APP/PS1 mice from inflammation and age-associated learning and memory deficits (Lewis et al., 2010). These changes were independent of APP processing, did not influence soluble or insoluble A β 40 or A β 42 levels or amyloid plaque burden (Anne M. Fagan et al., 2004; Lefterov et al., 2010; Lewis et al., 2010) and did not affect the levels of brain apoE (Anne M. Fagan et al., 2004; Lefterov et al., 2010), ABCA1 (Anne M. Fagan et al., 2004),

or cholesterol content (Lefterov et al., 2010; Lewis et al., 2010). However, APP/PS1 apoA-I^{-/-} mice showed a 10-fold increase in insoluble A β 40 and a 1.5-fold increase in A β 42 in cortical and hippocampal blood vessels, indicating that these mice have significantly increased cerebral amyloid angiopathy (CAA) (Lefterov et al., 2010). In contrast, levels of CAA were decreased by 44% in APP/PS1 mice over-expressing apoA-I (Lewis et al., 2010). These studies demonstrate that although apoA-I is not a major apolipoprotein in brain parenchyma, it nevertheless can influence inflammation, cognitive function and A β metabolism in the cerebral vasculature. Whether brain delivery of apoA-I can be used to stimulate apoE recycling and promote brain RCT now becomes an important question to pursue.

The relevance of this question is highlighted by recent findings on the importance of apoE genotype on recycling and brain physiology. ApoE4 is impaired in HDL-induced recycling of TRLs (Heeren et al., 2004). Furthermore, the low pH of lysosomes may preferentially affect the molten globule structure of apoE4 that retards its release into the recycling pathway (Hatters et al., 2005; Morrow et al., 2002; Zhong et al., 2009; Zhong & Weisgraber, 2009). ApoE3 binds HDL more readily than apoE4 (Dong et al., 1994), suggesting that it may be more efficiently stimulated by HDL or apoA-I to enter the recycling pathway. Recently, expression of apoE4 in neurons was shown to impair glutamate receptor function and apoer2 receptor recycling by sequestering NMDA, AMPA, and apoer2 receptors intracellularly, thereby reducing their cell surface expression (Y. Chen et al., 2010). As such, Reelin-induced long-term potentiation (LTP) is reduced, ultimately leading to impaired synaptic plasticity. Our results show that apoA-I stimulation of apoE recycling in glia increases LDLR levels in WT and ABCA1^{-/-} glia. Future

studies will address whether apoA-I can rescue impaired recycling of receptors in apoE4-expressing cells.

Another question raised by our study is whether stimulating apoE recycling may be beneficial or detrimental with respect to A β clearance and AD pathogenesis. On one hand, increasing apoE recycling may promote brain RCT and elevate glutamate receptor levels. Stimulation of recycling may correct the relative deficit of apoE4 in brain and CSF, as the net levels of apoE4 are lower in brain, CSF and plasma of human apoE4 targeted replacement mice compared to apoE2 and apoE4 controls (Riddell et al., 2008). Several other human and animal studies have also reported reduced apoE4 levels in brain (Beffert et al., 1999; Bertrand et al., 1995; Glockner et al., 2002; Poirier, 2005; Ramaswamy et al., 2005) but this is not always observed (Fryer et al., 2005b; Fukumoto et al., 2003; Sullivan et al., 2004). On the other hand, our data would suggest that recycled apoE emerges as a lipid-poor particle that, unless rapidly lipidated by ABCA1, would be expected to promote amyloidogenesis (Hirsch-Reinshagen et al., 2005; R. Koldamova et al., 2005). Finally, nothing is known about whether the various apoE lipoprotein particles secreted by glia vary in their interactions with A β and how such interactions may or may not affect lipoprotein function in RCT. Although much remains to be learned about how apoE functions in the healthy and diseased brain, continued activity in this field of research may offer ways to overcome the detrimental aspects of apoE4 for both acute and chronic neurological conditions.

Chapter 3: Hormonal modulators of glial ABCA1 and apoE levels

3.1 Introduction

Apolipoprotein E (apoE) is the major lipoprotein produced in the central nervous system (CNS), where it is secreted from astrocytes and microglia (Pitas et al., 1987a). Although CNS apoE does not intermingle with the pool of peripheral apoE produced by hepatocytes and macrophages (Linton et al., 1991), it performs similar functions in coordinating the transport of lipids among various cell types in the CNS (Ladu et al., 2000a; J. E. Vance & Hayashi, 2010). ApoE receives lipids from the cholesterol and phospholipid transporter ABCA1 and delivers its lipid cargo to recipient cells primarily through binding and endocytosis of the low-density lipoprotein receptor (LDLR) (Fryer et al., 2005a; Hirsch-Reinshagen et al., 2004). ApoE also binds several additional receptors including LDL-receptor-related protein (LRP), apolipoprotein E receptor 2 (apoER2) and VLDL receptor, which result in activation of signalling pathways important for neuronal function (Herz, 2009).

Humans possess three allelic isoforms of the 299 amino acid apoE protein: apoE2 (Cys112, Cys158), apoE3 (Cys112, Arg158) and apoE4 (Arg112, Arg158) (Strittmatter & Roses, 1995), and the human apoE sequence differs considerably from that of mice (Pennacchio & Rubin, 2003). ApoE4 increases Alzheimer's Disease (AD) risk and reduces age of onset of AD whereas apoE2 delays AD onset and reduces risk (Corder et al., 1994; Corder et al., 1993; Poirier et al., 1993). These effects are believed to be due in part to isoform-specific differences in A β metabolism, as apoE4 prolongs A β half-life in brain interstitial fluid (Cirrito et al., 2003) and promotes oligomerization of A β both in vivo and in vitro (Hashimoto et al., 2012). As apoE can

bind to A β primarily through interactions with the lipid-binding amphipathic α -helical region of apoE (Strittmatter et al., 1993b; Wisniewski & Frangione, 1992), the lipidation status of apoE may influence its interaction with A β . ApoE4 has a poor ability to accept lipids, as approximately twice as much cholesterol and phospholipid can be effluxed to apoE3 compared to apoE4 in cultured astrocytes (Michikawa et al., 2000). Also, uptake of apoE4 by neurons impairs glutamate receptor function and apoER2 receptor recycling, leading to reduced Reelin-induced long-term potentiation and dysfunctional synaptic plasticity (Y. Chen et al., 2010). ApoE^{-/-} mice also have worse outcomes after traumatic brain injury (TBI), spinal cord injury, stroke and ischemia (Y. Chen et al., 1997; Han & Chung, 2000; Laskowitz et al., 1997; Lynch et al., 2002), demonstrating the potential of apoE function to promote repair and recovery after a variety of acute brain injuries. Methods to improve apoE function are therefore of great interest as potential therapeutic approaches for both acute and chronic neurological conditions.

ABCA1 activity is a key regulator of apoE levels and function in the CNS (Hirsch-Reinshagen et al., 2004). ABCA1 deficiency leads to poorly lipidated and rapidly degraded apoE in the CNS (Hirsch-Reinshagen et al., 2004; Wahrle et al., 2004), which reduces apoE levels and increases amyloid burden in AD mice (Hirsch-Reinshagen et al., 2005; R. Koldamova et al., 2005; Wahrle et al., 2005). Conversely, selective overexpression of ABCA1 increases CNS apoE lipidation and markedly decreases amyloid deposition in AD mice (Wahrle et al., 2008). ABCA1-mediated lipidation of apoE promotes the proteolytic degradation of A β peptides (Q. Jiang et al., 2008). The most established agents that increase ABCA1 activity and enhance apoE lipidation are liver-X-receptor (LXR) agonists. LXR α/β are ligand-activated transcription factors of the nuclear hormone receptor superfamily that are activated by cholesterol-derived oxysterol ligands

(Beaven & Tontonoz, 2006; Janowski et al., 1996; Kalaany & Mangelsdorf, 2006; Lehmann et al., 1997). LXR α is enriched in liver, adipose tissue and macrophages, whereas LXR β is ubiquitously expressed (Willy et al., 1995). LXR target genes regulate cholesterol and lipoprotein metabolism as well as inflammation (Jamroz-Wisniewska et al., 2007; Wójcicka et al., 2007; Zelcer & Tontonoz, 2006). Notably, apoE has potent anti-inflammatory activities and may contribute to both lipid mobilization and reduced inflammation in response to LXR activation (Mahley & Rall, 2000). Consistent with the results obtained from selective loss of ABCA1, genetic deficiency of either LXR α or LXR β increases amyloid burden in AD mice (Zelcer et al., 2007).

Importantly, LXR agonists show considerable efficacy in maintaining cognitive function in several AD mouse models (Donkin et al., 2010; Fitz et al., 2010; R. P. Koldamova et al., 2005; Riddell et al., 2007). Synthetic LXR agonists including TO901317 and GW3965 cross the blood brain barrier and stimulate expression of many target genes including ABCA1 and apoE. Treatment of multiple AD mouse models with TO901317 or GW3965 consistently improves memory and reduces A β levels (Donkin et al., 2010; Fitz et al., 2010; R. P. Koldamova et al., 2005; Riddell et al., 2007). Importantly, 7 days of TO901317 treatment is sufficient to produce cognitive benefits and increase cortical ABCA1 levels (Donkin et al., 2010; Riddell et al., 2007), demonstrating that LXR agonists may also be useful for acute indications. We recently showed that ABCA1 is required for several beneficial effects of GW3965 in APP/PS1 mice including increased CSF apoE protein, reduced amyloid load and improved memory (Donkin et al., 2010). These results suggest that increasing apoE lipidation may underlie some of the neuroprotective effects of LXR agonists. However, despite their efficacy and tolerability in rodents, current LXR

agonists have a significant caveat that currently precludes their translation into human clinical trials. Specifically, these compounds also activate liver sterol response element binding protein 1c (SREBP-1c) and fatty acid synthase (FAS), which rapidly leads to hypertriglyceridemia and hepatic steatosis in species such as humans that express cholesteryl ester transfer protein (Groot et al., 2005). As a result, the therapeutic potential of LXR agonists remains untapped and alternative methods to enhance apoE function in the brain remains an important endeavor.

To identify alternative pathways by which apoE is regulated, we performed a high throughput screen (HTS) for compounds that increase apoE secretion from human CCF-STTG1 astrocytoma cells. Here we report that apoE secretion from CCF-STTG1 cells is significantly enhanced by the synthetic progestin lynestrenol and moderately stimulated by progesterone. Intriguingly, progesterone and lynestrenol use multiple mechanisms to promote apoE secretion in CCF-STTG1 astrocytoma cells. Compared to GW3965, lynestrenol is a substantial LXR agonist with respect to ABCA1 induction, progesterone has weaker LXR activity and the progesterone metabolite allopregnanolone has none. In CCF-STTG1 cells, progesterone receptor (PR) inhibition attenuates the ability of lynestrenol and progesterone to increase apoE expression but does not affect ABCA1 expression. Lynestrenol also stimulates ABCA1 expression in primary murine glia and immortalized murine astrocytes that express human apoE3. Our observations offer new insights into how specific reproductive hormones may affect glial lipid homeostasis.

3.2 Materials and methods

3.2.1 Cell lines and reagents

Human CCF-STTG1 astrocytoma cells and human hepatoma HepG2 cells were purchased from ATCC (Manassas, VA). Immortalized LXR-double-knockout ($LXR\alpha^-/LXR\beta^-$) and $LXR\alpha$ -expressing ($LXR\alpha^+$) mouse embryonic fibroblasts (MEFs) have been described (W. K. Kim et al., 2009). Astrocytes derived from human apoE3 or apoE4 knock-in mice that were immortalized by SV40 T antigen were obtained from Dr. David Holtzman (Morikawa et al., 2005). GW3965 was provided by Dr. Jon Collins (GlaxoSmithKline, NC). Estrone, 17α -estradiol, 17β -estradiol, estriol, RU486 (Mifepristone) and recombinant human apoE3 were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human apoA-I was purchased from Calbiochem (now Millipore, Billerica, MA). Progesterone, allopregnanolone and lynestrenol were purchased from Steraloids, Inc. (Newport, RI). Stocks of GW3965 (1 mM), RU486 (5 mM), 17β -estradiol (10 mM), progesterone (1 mM), allopregnanolone (6.25 mM) and lynestrenol (1 mM) were prepared in dimethyl sulfoxide (DMSO).

3.2.2 Cell culture and treatment

CCF-STTG1 and MEF cells were cultured in growth media consisting of Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Gibco), 2 mM L-glutamine and 1% penicillin/streptomycin (Invitrogen). Immortalized human apoE3- and apoE4-expressing astrocytes were maintained in the above growth media supplemented by 200 μ g/ml Geneticin and 1 mM sodium pyruvate. HepG2 cells were cultured in the same growth media supplemented with 1mM sodium pyruvate and 1x non-essential amino acids (Invitrogen). Primary mixed glia were prepared from post-natal day 0-2 pups exactly as described (J. Fan et al., 2011). Glial cells

were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Gibco), 2 mM L-glutamine and 1% penicillin/streptomycin (Invitrogen). Media was changed every 7 days until cells were fully confluent at approximately 21 days, followed by re-seeding for experiments as described below.

For dose-dependency experiments, CCF-STTG1 cells were seeded in 384-well plates at 17,500 cells/well in growth media, incubated for 2 h, and then treated with the reference compound GW3965 (800 nM) or test compounds (0.3125 – 40 μ M) for 96 h. Media was collected, centrifuged at 1,000 x g for 3 min and apoE was quantified by ELISA. For time course experiments, CCF-STTG1 cells were seeded in 12-well plates (BD Falcon) in growth media at 400,000 cells/well. After 24h, cells were washed once with serum-free DMEM:F12 media (Gibco) and treated with DMSO alone as a negative control, 1 μ M of GW3965 as a positive control, or 10 μ M of test compounds in DMEM:F12 media containing 2% FBS and 1% penicillin/streptomycin for 24, 48, 72 and 96h. For immunoblotting and mRNA analyses, CCF-STTG1 cells were seeded in 12-well plates at 450,000 cells/well and MEFs were seeded in 12-well plates at 80,000 cells/well. After 24 h, cells were washed once with serum-free conditioning media consisting of 1:1 DMEM:F12 with 1% penicillin/streptomycin and treated with DMSO alone, 1 μ M GW3965, or test compounds (1-10 μ M) in 800 μ l/well of such conditioning media for another 24 h. HepG2 cells were seeded in 12-well plates at 400,000 cells/well 24h before treatment. Cells were then treated with DMSO alone, 1 μ M GW3965, or test compounds (1-10 μ M) in the above serum-free media for another 24h. The final concentration of DMSO was equalized in all treatment conditions. At the end of the treatment period, conditioned media was collected and centrifuged at 1,200 x g for 3 min to remove cell debris. Cells were washed twice

with 1x PBS and lysed either in 150 μ l/well of radioimmunoprecipitation assay (RIPA) lysis buffer (20 mM Tris, 1% NP40, 5 mM EDTA, 50 mM NaCl, 10 mM Na pyrophosphate, 50 mM NaF, and complete protease inhibitor, pH 7.4) for cellular protein analysis, or in 800 μ l/well of Trizol (Invitrogen) for mRNA analysis. Samples were stored at -80°C until analyzed.

3.2.3 Cholesterol efflux assay

CCF-STTG1 cells were seeded at 250,000 cells/well in 24-well plates and labeled for 24 h with 1 μ Ci/ml of 3H-Cholesterol (PerkinElmer Life Sciences) in growth media supplemented by DMSO only (control), 1 μ M of GW3965, 10 μ M of lynestrenol or 10 μ M progesterone. Labeled cells were then washed and equilibrated in serum free DMEM:F12 for 1 h. Serum-free DMEM:F12 media containing the same drug treatments were then added to the cells in the absence (NA, no acceptor) or presence of 5 μ g/ml of exogenous lipid-free apoA-I (Calbiochem) or apoE3 (Sigma) for 8 h. Media was collected and centrifuged at 8000 rpm for 3 min. Cells were lysed by addition of 0.1M NaOH and 0.2% SDS followed by incubation at room temperature for 30 min.

Radioactivity in media and cell lysate samples was quantified by scintillation counting (PerkinElmer). The percent cholesterol efflux was calculated as the total counts per minute (CPM) in the media divided by the sum of the CPM in the media plus the cell lysate, multiplied by 100.

3.2.4 Progesterone receptor inhibition

Cells were seeded in 12-well plates at the following densities: 450,000 cells/well for CCF-STTG1 cells, 100,000 cells/well for immortalized human-apoE3-astrocytes and 80,000 cells/well for apoE4-expressing astrocytes. After 24 h in growth media, cells were washed with serum-free

conditioning media and pre-treated with or without 5 μ M RU486 in serum-free media for 1 h. Cells were then treated with 1 μ M of GW3965 or 10 μ M of test compounds in serum-free conditioning media for CCF-STTG1, or 1 μ M of test compounds in conditioning media containing 2% FBS for human-apoE-expressing astrocytes, in the presence or absence of 5 μ M RU486 for another 24 h, followed by collection of media and cells. Primary mixed glial cells were re-seeded in 12-well plates and maintained in growth media for 4-5 days until confluent. Primary glial cells were washed and treated in serum-free conditioning media with the same dosage of drugs as above except that the treatment period was 72h.

3.2.5 ApoE ELISA

For dose dependency tests, ELISA plates (384-well, ThermoScientific) were coated with anti-apoE capture antibody (Abcam, cat # ab7620) at 2.5 μ g/ml in PBS at 4°C overnight, washed four times with 100 μ l wash buffer (0.05% Tween 20 in PBS) using a plate washer (BioTek Instruments) and blocked for 1 h at RT using 1% bovine serum albumin in PBS (blocking buffer) dispensed with a Microfill microplate dispenser (BioTek Instruments). Media samples or standards (25 μ l) were added to blocked wells using a Biomek FX laboratory automation workstation and incubated for 1.5 h at RT. After washing four times as above, 25 μ l of 500 ng/ml anti-apoE detection antibody (Abcam, cat # ab20261) in blocking buffer was added, incubated for 1 h at RT, and washed four times as above. Peroxidase substrate (TMB, Sigma-Aldrich, 25 μ l) was then added, incubated for 15 min in the dark, followed by adding 25 μ l of stop reagent (TMB substrate, Sigma-Aldrich) using the Wellmate Dispenser. Plates were immediately read at 450 nm absorbance. For analysis of media time course experiments, a commercial ELISA kit was used (MBL, cat # 7635). In this case, unconcentrated media samples were diluted with assay

diluent solution provided by the kit and measured according to the manufacturer's instruction. Plates were read at 450 nm absorbance.

3.2.6 Immunoblotting

RIPA cellular lysates were sonicated in a Branson 1510 water-bath sonicator (Branson Ultrasonics, Danbury, CT) for 10 min. Protein concentration was determined by Lowry assay (Bio-Rad). Cellular proteins (20-40 µg/well) were mixed with loading dye containing 2% SDS, 1% β-mercaptoethanol, 5U/µl DNaseI (Invitrogen). Unconcentrated conditioned media (40µl/lane) was mixed with loading dye containing 2% SDS and 1% β-mercaptoethanol. Samples were incubated for 5 min at 90°C and resolved on 10% Tris-HCl polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride (PVDF, Millipore) membranes at 24 V overnight at 4°C. After blocking with 5% non-fat milk in PBS for 1 h, membranes were probed overnight at 4°C with 1:1000 goat-anti human apoE (Chemicon, cat# AB947) or 1:1000 goat-anti murine apoE (Santa Cruz), 1:2000 monoclonal anti-ABCA1 (a gift from Dr. Michael Hayden), 1:500 goat-anti human LDLR (R&D Systems) or 1:1000 goat-anti murine LDLR (R&D Systems), or probed for 1 h at RT with 1:2000 rabbit-anti-LRP (a gift from Dr. Joachim Herz) or 1:5000 anti-β-actin or anti-GAPDH. Membranes were washed with 2xPBS-T (2xPBS with 0.05% Tween-20) 4 times for 8 min each and incubated for 1 h with horseradish peroxidase (HRP)-labeled anti-goat (1:1000) or anti-mouse (1:1000 for ABCA1 detection, 1:5000 for actin or GAPDH detection) secondary antibodies (Jackson ImmunoResearch). Results were visualized using chemiluminescence (ECL, Amersham). Films were scanned and band density was determined using ImageJ (version 1.46, NIH) software. Levels of ABCA1, apoE, LDLR, LRP

were normalized to actin or GAPDH and expressed as fold difference compared to vehicle controls.

3.2.7 Quantitative RT-PCR

RNA was extracted using Trizol and treated with DNaseI according to the manufacturer's protocol. cDNA was generated using oligo-dT primers and Taqman reverse transcription reagents (Applied Biosystems, Foster City, CA). Quantitative real-time PCR primers were designed using PrimerExpress (Applied Biosystems) to span human-specific or murine-specific regions of ABCA1, SREBP-1c and apoE. Primer sequences and cycling conditions are available upon request. Real-time quantitative PCR was done with SYBR Green reagents (Applied Biosystems) on an ABI 7000 (Applied Biosystems). Each sample was assayed at least in duplicate, normalized to GAPDH and analyzed with 7000 system SDS software version 1.2 (Applied Biosystems) using the relative standard curve method.

3.2.8 Statistics

Data are shown as mean \pm standard deviation (SD) of the indicated number of independent experiments and analyzed by one-way ANOVA with a Tukey's Multiple Comparison post test, or by two-way ANOVA with a Sidak's and Tukey's post test. All statistical analyses were performed using GraphPad Prism (version 6.0; San Diego, CA).

3.3 Results

3.3.1 Estrogens have a negligible effect on apoE secretion from CCF-STTG1 astrocytoma cells.

Previous studies have shown that estrogens and progesterone have neuroprotective properties (Brinton et al., 2008). To determine whether estrogens can directly stimulate apoE secretion from astrocytes, we treated CCF-STTG1 human astrocytoma cells with estrone, 17 α -estradiol, 17 β -estradiol and estriol over a dose range of 0.3125 – 40 μ M for 96 h and evaluated secreted apoE levels by ELISA. Estrone, 17 α -estradiol and estriol at 0.3125 – 20 μ M showed no effect on apoE secretion (**Fig. 3.1A, B, D**, $p > 0.05$). Although statistically significant, apoE secretion was increased only by 1.32 or 1.29-fold with 2.5 or 5 μ M of 17 β -estradiol, respectively (**Fig. 3.1C**, $p < 0.01$, $p < 0.05$). All four estrogens at 40 μ M significantly decreased secreted apoE levels, possibly due to cellular toxicity (**Fig. 3.1**, $p < 0.0001$).

As low concentrations of 17 β -estradiol were previously reported to increase apoE production from mixed neuronal cultures (Nathan et al., 2004), we next tested a lower dose range (1 pM - 10 μ M) of 17 β -estradiol on CCF-STTG1 cells. After 24 h of treatment, no change in secreted apoE levels in media was observed by immunoblotting (See Appendix A supplemental Fig. 1).

Cellular protein levels of apoE and ABCA1 were also unaffected by 17 β -estradiol (See Appendix A supplemental Fig. 1), indicating that 17 β -estradiol does not exhibit LXR agonist activity in CCF-STTG1 human astrocytoma cells.

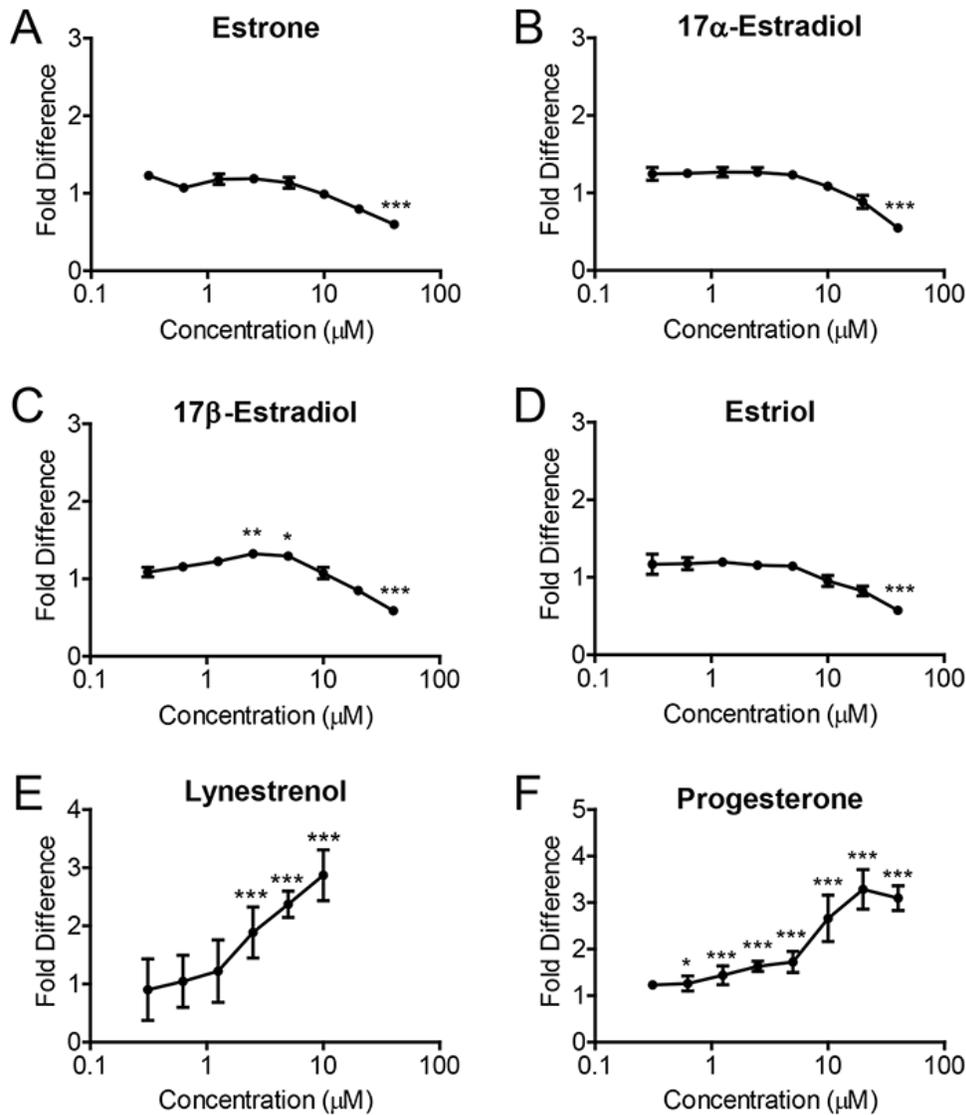


Figure 3.1: Estrogens do not increase apoE secretion from CCF-STTG1 cells, whereas lynestrenol and progesterone increase apoE secretion in a dose dependent manner.

CCF-STTG1 cells were seeded in 384-well plates at 17.5K cells/well and treated with 0.3125 to 40 μM of (A) estrone, (B) 17α-estradiol, (C) 17β-estradiol, (D) estriol, (E) lynestrenol, or (F) progesterone for 96h. Data represent fold difference of secreted media apoE, measured by ELISA, compared to the vehicle control (not shown). Graphs represent mean and SD of quadruplicate wells, analyzed by one-way ANOVA with a Tukey's post test. * represents $p < 0.05$, ** represents $p < 0.01$, and *** represents $p < 0.0001$.

3.3.2 Lynestrenol and progesterone increase apoE secretion from CCF-STTG1 astrocytoma cells.

To determine whether natural progesterone or synthetic progestins stimulate apoE secretion from astrocytoma cells, we treated CCF-STTG1 cells with progesterone and the progestins lynestrenol, medroxyprogesterone, D-norgestrel, chlormadine acetate, cyproterone acetate and 19-norethindrone at 0.3125 - 40 μ M for 96 h and evaluated secreted apoE levels by ELISA. Of these, only lynestrenol and progesterone significantly increased apoE secretion in a dose-dependent manner, with a maximum increase of 2.87-fold with 10 μ M lynestrenol (**Fig. 3.1E**, $p < 0.0001$) and a maximum increase of 3.28-fold with 20 μ M progesterone (**Fig. 3.1F**, $p < 0.0001$).

We then quantified the increase in secreted apoE levels after exposure to 10 μ M progestins over 24-96 h. Quantification by immunoblot (**Fig. 3.2A**) showed that by 24 h, similar to 1 μ M GW3965 (5.14-fold, $p < 0.01$), lynestrenol increased secreted apoE levels by 3.93-fold (**Fig. 3.2B**, $p < 0.05$), while a trend increase of 2.45-fold was found for progesterone. When quantified by denaturing immunoblot, 10 μ M lynestrenol consistently led to a significant increase in secreted apoE by approximately 4-fold relative to the negative control ($p < 0.05-0.01$), compared to an average 5.50-fold increase induced by 1 μ M GW3965 at each time point ($p < 0.01-0.001$) (**Fig. 3.2B**). Progesterone exhibited a modest trend toward elevated media apoE levels, although this did not reach statistical significance, whereas the progesterone metabolite allopregnanolone had negligible activity (**Fig. 3.2B**). Quantification of the same media samples by ELISA revealed that progesterone and allopregnanolone led to consistent trends of an average of 1.43-fold and 1.13-fold induction of apoE, respectively, at each time point, neither of which was significantly above

vehicle controls (**Fig. 3.2C**, left panel, $p>0.05$). By contrast, ELISA quantitation showed that 10 μM lynestrenol increased media apoE by 2.30-fold at 24h, 4.92-fold at 48h, 7.37-fold at 72h, and 11.20-fold at 96h compared to vehicle controls (**Fig. 3.2C**, right panel). By 96h, the 11.2-fold increase in secreted apoE detected by ELISA reached statistical significance (**Fig. 3.2C**, $p<0.01$).

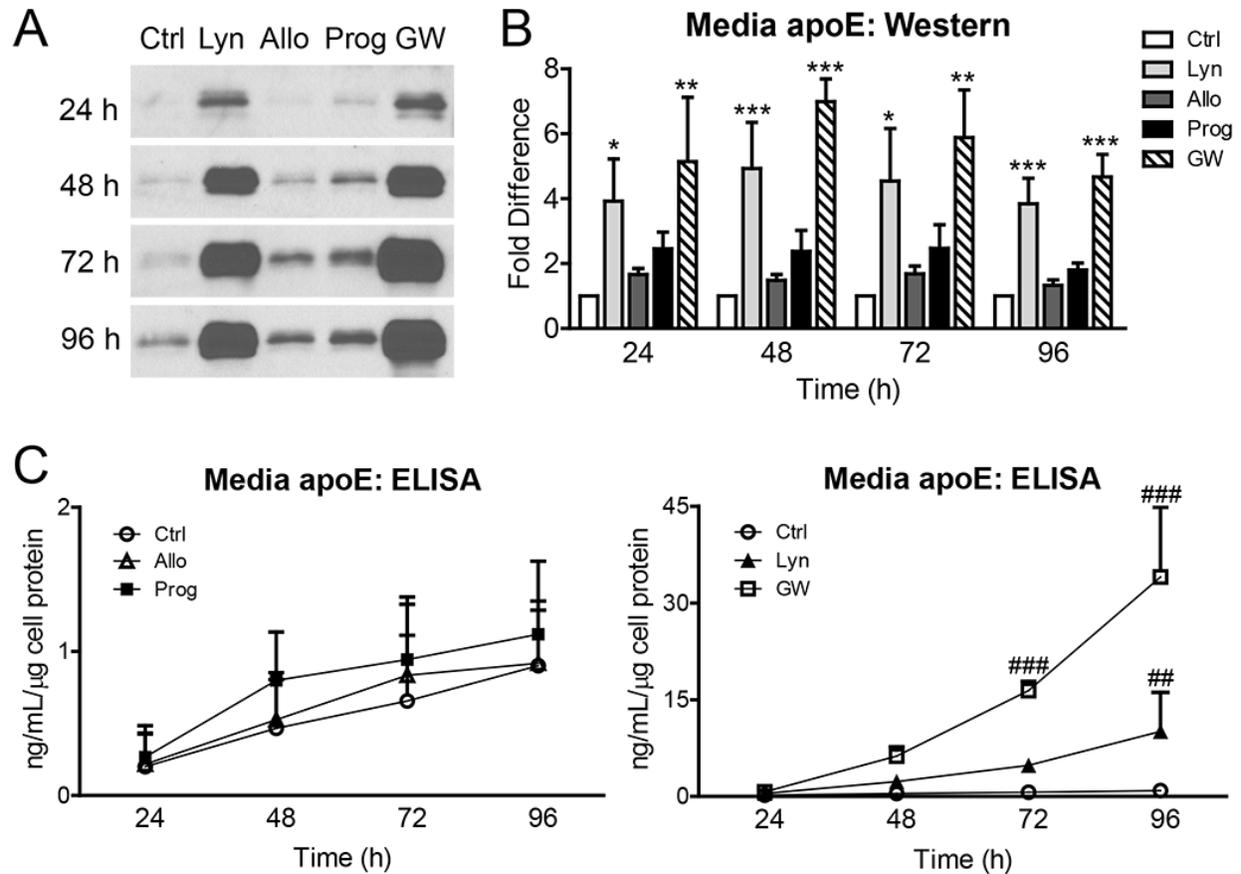


Figure 3.2: Time course of progestin-stimulated apoE secretion in astrocytoma.

CCF-STTG1 cells were treated with DMSO alone (Ctrl), 10 μ M each of lynestrenol (Lyn), allopregnanolone (Allo), progesterone (Prog), or 1 μ M of GW3965 (GW) for indicated time period (24h-96h). (A) Representative Western blots at 24, 48, 72 and 96h. (B) Band intensity was quantitated by densitometry and normalized against control bands from each respective time point. Data are expressed as the fold difference of media apoE normalized against total cellular protein in each well and analysed by one-way ANOVA with a Tukey's post test. * represents $p < 0.05$, ** represents $p < 0.01$, and *** represents $p < 0.0001$, compared to the control from each time point. (C) ApoE levels in media were quantified by ELISA, normalized against total cellular protein, and analysed by two-way ANOVA with a Tukey's post test. ## represents $p < 0.01$, and ### represents $p < 0.0001$, compared to the control from each time point. Data in B and C represent the mean and SD from three independent experiments each performed in duplicate.

3.3.3 Lynestrenol increases apoE, ABCA1 and SREBP-1c mRNA levels in CCF-STTG1 astrocytoma cells but is less effective for SREBP-1c activation in HepG2 hepatoma cells.

We and others have previously shown that ABCA1-mediated lipidation of apoE provides significant cognitive and biochemical benefits in AD mouse models (Donkin et al., 2010; Fitz et al., 2010; R. P. Koldamova et al., 2005; Riddell et al., 2007). To determine whether natural and synthetic progestins may stimulate this beneficial ABCA1-apoE pathway, we treated CCF-STTG1 cells with 1 or 10 μ M of lynestrenol, allopregnanolone and progesterone for 24 h, followed by measurement of mRNA levels by qRT-PCR. As expected, GW3965 increased apoE mRNA levels by 3.22-fold (**Fig. 3.3A**, $p < 0.0001$). Among the progestins, only 10 μ M lynestrenol significantly induced apoE mRNA levels by 1.99-fold (**Fig. 3.3A**, $p < 0.05$). In parallel, ABCA1 mRNA levels were significantly upregulated by GW3965 (12.77-fold) and 10 μ M lynestrenol (12.54-fold) compared to control (**Fig. 3.3B**, $p < 0.0001$), while a trend increase was observed with 10 μ M progesterone (3.75-fold). The response of ABCA1 mRNA to 10 μ M lynestrenol was also significantly higher than with 1 μ M lynestrenol (**Fig. 3.3B**, $p < 0.0001$). A third LXR-target gene, SREBP-1c, was then examined. SREBP-1c mRNA levels were significantly increased by GW3965 (5.58-fold) and 10 μ M lynestrenol (5.31-fold) (**Fig. 3.3C**, $p < 0.0001$), while progesterone showed a trend toward an increase (3.59-fold). Both lynestrenol and progesterone significantly increased SREBP-1c mRNA levels in a dose-dependent manner in CCF-STTG1 cells (**Fig. 3.3C**, $p < 0.01$ for lynestrenol and $p < 0.05$ for progesterone). By contrast, allopregnanolone had no significant effect on ABCA1, apoE or SREBP-1c mRNA levels (**Fig. 3.3**, $p > 0.05$). Together, these results suggest that lynestrenol has moderate activity as an LXR agonist, whereas progesterone exhibits weak LXR agonist activity and allopregnanolone does not activate LXR in CCF-STTG1 cells.

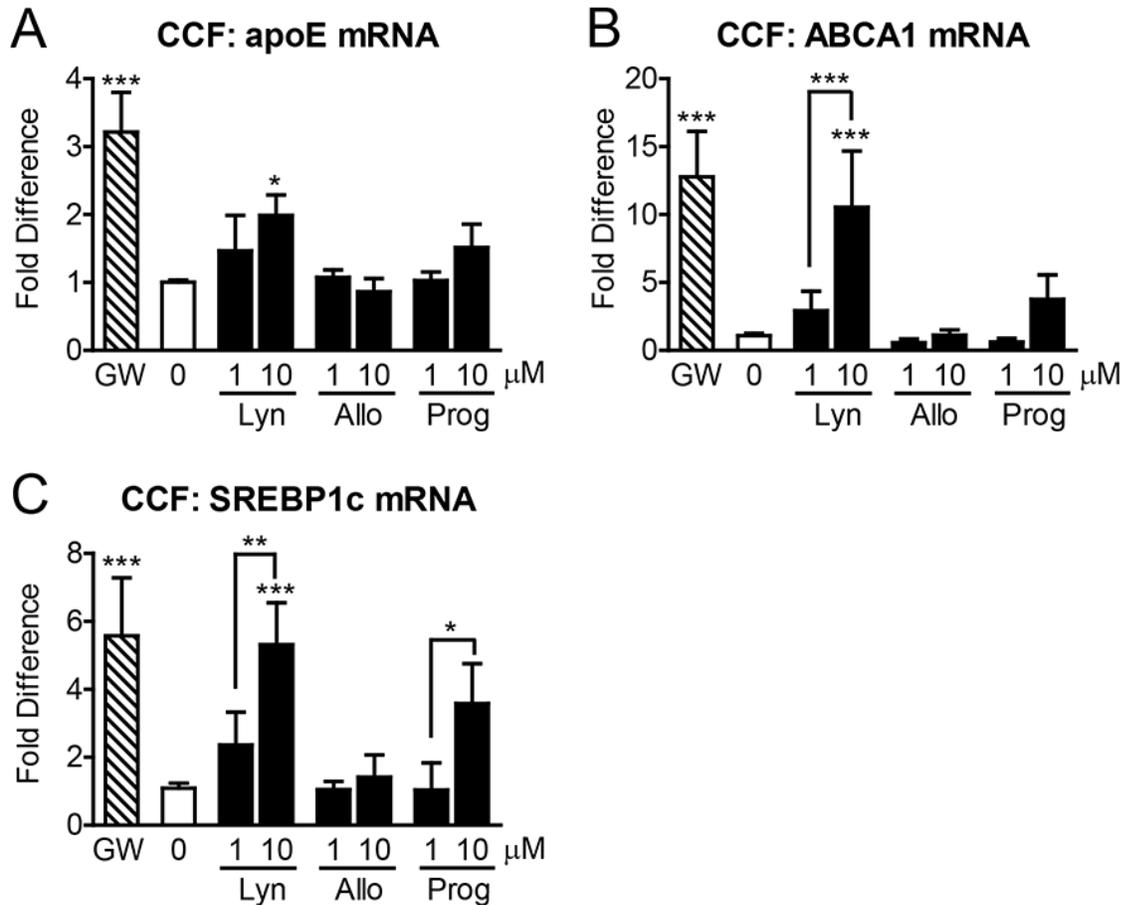


Figure 3.3: Lynestrenol and progesterone increase apoE, ABCA1 and SREBP-1c mRNA levels in astrocytoma cells.

CCF-STTG1 cells were treated with DMSO alone (0), 1 μM of GW3965 (GW), 1 or 10 μM each of lynestrenol (Lyn), allopregnanolone (Allo) and progesterone (Prog) for 24h. Real-time quantitative PCR was used to measure apoE (A), ABCA1 (B) and SREBP-1c (C) mRNA levels in whole cell lysates. Data represent mean and SD of four independent experiments, analysed by one-way ANOVA with a Tukey's post test. * p<0.05, ** p<0.01, *** p<0.0001.

As induction of SREBP-1c-mediated lipogenesis in the liver is a major reason why current LXR agonists have not advanced into clinical trials, we next evaluated the effects of lynestrenol and progesterone on ABCA1 and SREBP-1c mRNA levels in HepG2 cells. Treatment of HepG2 cells with 1 μ M GW3965 led to a 6.46-fold induction of ABCA1 mRNA (**Fig. 3.4A**, $p < 0.05$), whereas neither lynestrenol nor progesterone significantly elevated ABCA1 mRNA levels even at 10 μ M (**Fig. 3.4A**, $p > 0.05$), which is consistent with the established weak response of ABCA1 to LXR agonists in hepatic cells. Intriguingly, SREBP-1c levels were induced by 19.25-fold by 1 μ M GW3965 ($p < 0.0001$) but only 7.32-fold by 10 μ M lynestrenol ($p < 0.05$) and 3.39-fold by 10 μ M progesterone which was not statistically significant when analysed by one-way ANOVA (**Fig. 3.4B**). These observations suggest that there may be cell-type specific co-regulators that differ between CCF-STTG1 and HepG2 cells that could result in a more favorable safety profile of lynestrenol compared to GW3965.

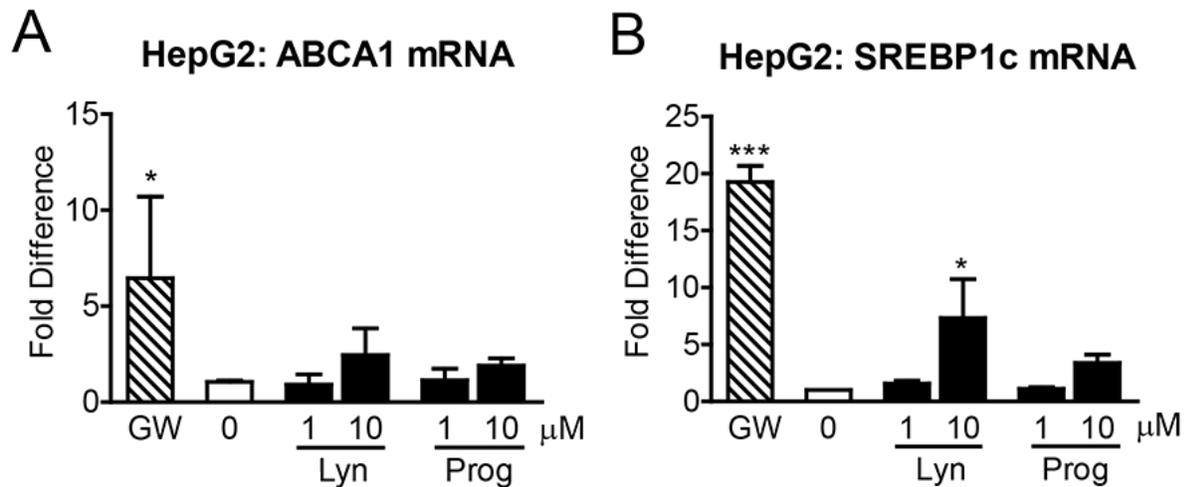


Figure 3.4: Lynestrenol and progesterone do not significantly activate SREBP-1c mRNA levels in HepG2 cells.

HepG2 cells were treated with DMSO alone (0), 1 μ M of GW3965 (GW), 1 or 10 μ M each of lynestrenol (Lyn) and progesterone (Prog) for 24h. Real-time quantitative PCR was used to measure ABCA1 (A) and SREBP-1c (B) mRNA levels in whole cell lysates. Data represent mean and SD of four independent experiments for A and two experiments for B, analysed by one-way ANOVA with a Tukey's post test. * $p < 0.05$, *** $p < 0.0001$.

3.3.4 Lynestrenol increases ABCA1 protein levels in CCF-STTG1 astrocytoma cells.

To determine the effect of these natural and synthetic progestins on the levels of proteins involved in cellular lipid homeostasis, we next evaluated the response of ABCA1, apoE, LDLR and LRP by immunoblotting (**Fig. 3.5A**). Unlike the LXR agonist GW3965, none of the hormones tested significantly increased cellular apoE protein levels (**Fig. 3.5B**). 10 μ M lynestrenol induced a significant 4.25-fold increase in ABCA1 protein levels over baseline, which was roughly comparable to the 5.60-fold increase induced by GW3965 (**Fig. 3.5C**, $p < 0.0001$). Although 10 μ M progesterone increased ABCA1 protein levels by 3.75-fold compared to baseline, this increase was not statistically significant (**Fig. 3.5C**, $p > 0.05$). Allopregnanolone had no significant effect on ABCA1 protein levels (**Fig. 3.5C**, $p > 0.05$). None of the compounds tested had a significant effect on cellular LDLR or LRP levels (**Fig. 3.5D, E**, $p > 0.05$), although an intriguing trend toward decreased LDLR levels was observed with 10 μ M progesterone (**Fig. 3.5D**). This observation suggests that inhibition of apoE uptake and/or recycling may contribute to the ability of progesterone to increase media apoE levels.

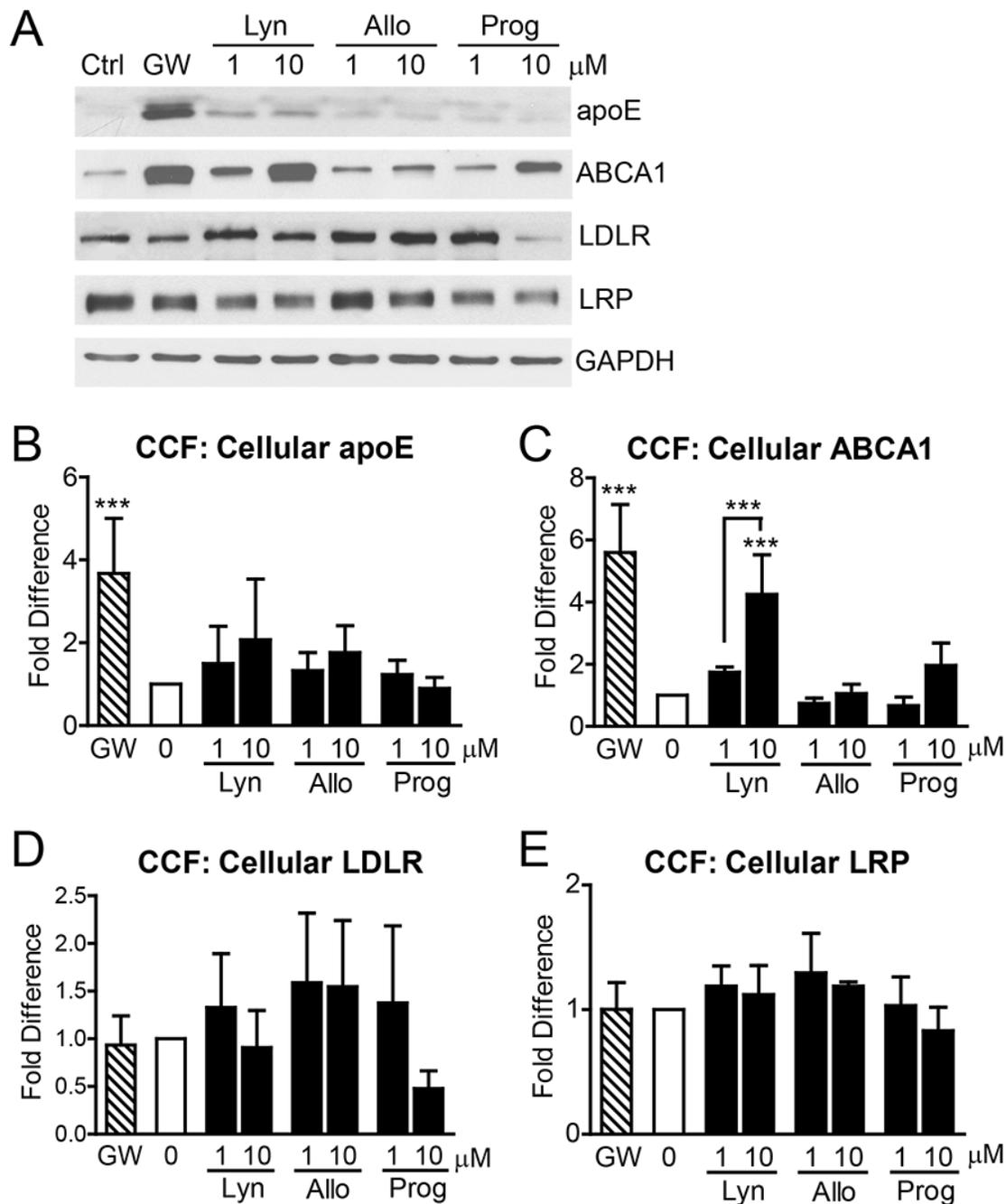


Figure 3.5: Lynestrenol increases ABCA1 protein levels in astrocytoma cells.

CCF-STTG1 cells were treated with DMSO alone (0), 1 μM of GW3965 (GW), 1 or 10 μM each of lynestrenol (Lyn), allopregnanolone (Allo) and progesterone (Prog) for 24h. Western blotting (A) was used to measure cellular protein levels of apoE (B), ABCA1 (C), LDLR (D) and LRP (E) in whole cell lysates. Graphs represent mean and SD of five independent experiments for B and C, four experiments for D and three experiments for E, analysed by one-way ANOVA with a Tukey's post test. *** represents $p < 0.0001$.

3.3.5 Lynestrenol enhances apoA-I and apoE3-mediated cholesterol efflux from CCF-STTG1 astrocytoma cells.

To determine the effect of progesterone and lynestrenol on ABCA1 function, we measured cholesterol efflux over an 8 h period to exogenous unlipidated recombinant apoA-I and apoE3 in CCF-STTG1 cells that were co-treated with test compounds. The effect of different progestins and different apolipoprotein acceptors on cholesterol efflux in astrocytoma cells is illustrated in **Fig. 3.6**. As expected, GW3965 significantly induced apoA-I and apoE3-mediated cholesterol efflux compared to non-acceptor (NA) conditions (apoA-I: $4.23 \pm 0.57\%$, apoE3: $3.82 \pm 0.56\%$ vs. NA: $1.32 \pm 0.21\%$, $p < 0.0001$ for apoA-I vs. NA, $p < 0.01$ for apoE3 vs. NA). Interestingly, lynestrenol increased apoA-I and apoE3-mediated efflux to a greater extent than GW3965. Specifically, lynestrenol significantly increased cholesterol efflux from $2.83 \pm 0.47\%$ at baseline to $12.61 \pm 2.47\%$ in the presence of apoA-I and to $5.52 \pm 1.43\%$ in the presence of apoE3 (each $p < 0.0001$). These results indicate that lynestrenol increases ABCA1 activity.

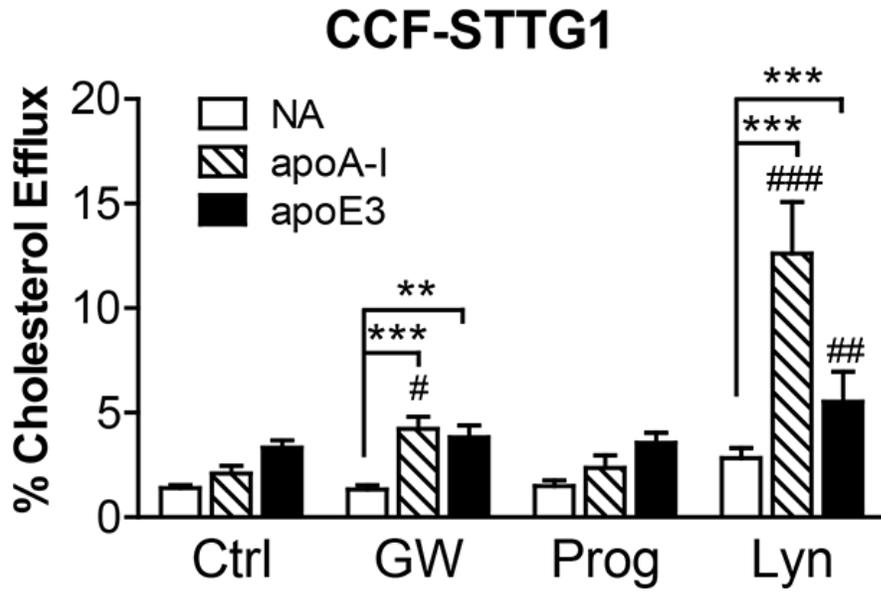


Figure 3.6: Cholesterol efflux is enhanced by lynestrenol in CCF-STTG1 cells.

CCF-STTG1 cells were seeded in 24-well plates and incubated in 3H-cholesterol containing media with co-treatment of DMSO alone (Ctrl), 1 μ M of GW3965 (GW), 10 μ M of lynestrenol (Lyn) or 10 μ M of progesterone (Prog) for 24h. Cholesterol efflux over 8h in the absence (NA) or presence of 5 μ g/ml of exogenous apoA-I or apoE3 along with the drug treatment was evaluated. Graphs represent means and SD of four independent experiments each conducted in duplicate. Two-way ANOVA with a Tukey's post test (comparing rows) was used to determine the drug effect over respective baselines, with # presenting $p < 0.05$, ## representing $p < 0.01$, and ### representing $p < 0.0001$. Another Tukey's post test (comparing columns) was used to determine the acceptor effect, with ** representing $p < 0.01$ and *** representing $p < 0.0001$.

3.3.6 ABCA1 induction by lynestrenol and progesterone is LXR-dependent.

Because the activity of lynestrenol and progesterone partly resembles that of the known LXR agonist GW3965, we next assessed ABCA1 mRNA levels in LXR α expressing (LXR α +) and LXR α / β DKO (LXR α -/LXR β -) MEF cells treated with 10 μ M lynestrenol, allopregnanolone and progesterone. In LXR α -expressing MEFs, ABCA1 mRNA levels were significantly increased by GW3965 and lynestrenol by 6.38-fold ($p < 0.0001$) and 5.58-fold ($p < 0.01$), respectively, compared to vehicle control. Progesterone resulted in a 3.18-fold increase of ABCA1 mRNA that did not reach statistical significance (**Fig. 3.7**, $p = 0.17$). As expected, loss of LXR α / β greatly impeded the ability of GW3965 to induce ABCA1 mRNA (**Fig. 3.7**, $p < 0.0001$, two-way ANOVA with a Sidak's post test). Similarly, loss of LXR α / β also blocked the ability of lynestrenol ($p < 0.0001$) and progesterone ($p < 0.05$) to stimulate ABCA1 transcription in MEFs (**Fig. 3.7**, two-way ANOVA with a Sidak's post test). These results demonstrate that lynestrenol and progesterone activate ABCA1 expression through LXR-dependent pathways. Consistent with the results in CCF-STTG1 cells, allopregnanolone had no significant influence on ABCA1 mRNA levels with or without functional LXR, confirming that allopregnanolone has no activity as an LXR agonist (**Fig. 3.7**).

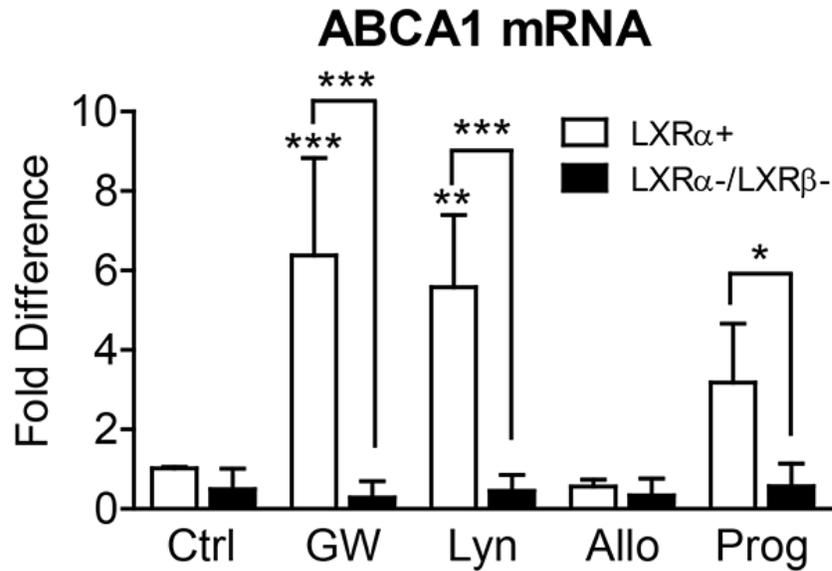


Figure 3.7: Induction of ABCA1 mRNA level by lynestrenol and progesterone is LXR dependent.

LXR α expressing (LXR α +) and LXR α / β DKO (LXR α -/LXR β -) MEF cells were treated with DMSO alone (Ctrl), 1 μ M of GW3965 (GW), 10 μ M of lynestrenol (Lyn), allopregnanolone (Allo) and progesterone (Prog) for 24h. ABCA1 mRNA level in whole cell lysate was measured by real-time quantitative PCR and presented in fold difference to the control group from LXR □ expressing cells. The effect of drug treatments, compared to the control within each genotype, was analysed by two-way ANOVA with a Tukey's post test. The difference of response to each drug treatment between two genotypes was compared using two-way ANOVA with a Sidak's multiple comparisons test. Data illustrate mean and SD from three independent experiments. * represents $p < 0.05$, ** represents $p < 0.01$, and *** represents $p < 0.0001$.

3.3.7 Upregulation of apoE by lynestrenol and progesterone in CCF-STTG1 astrocytoma cells also involves the progesterone receptor.

To investigate whether progesterone receptor (PR) activity affects apoE induction by lynestrenol or progesterone, CCF-STTG1 cells were pre-treated with DMSO or 5 μ M of the PR inhibitor RU486 for 1 h, followed by treatment with vehicle control, 1 μ M GW3965, 10 μ M lynestrenol or 10 μ M progesterone with or without 5 μ M RU486 for another 24 h. Secreted apoE and cellular ABCA1 and apoE protein levels were determined by immunoblotting (**Fig. 3.8A**).

RU486 alone had no significant effect on secreted apoE (**Fig. 3.8B**), cellular apoE (**Fig. 3.8C**) or cellular ABCA1 protein (**Fig. 3.8D**, black versus white bar in control (Ctrl) group, $p>0.05$). As expected, lynestrenol and GW3965 significantly increased media apoE in the absence of RU486 (**Fig. 3.8B**, $p<0.05$ and $p<0.01$, respectively). Induction of secreted apoE by GW3965 was maintained in the presence of RU486 (**Fig. 3.8B**, $p<0.01$), demonstrating that RU486 does not inhibit the LXR pathway activated by GW3965. However, RU486 blocked the ability of lynestrenol to significantly increase secreted apoE from baseline and also attenuated the trend toward increased apoE secretion by progesterone (**Fig. 3.8B**). RU486 also inhibited the trend toward increased cellular apoE levels by progesterone and lynestrenol but had no effect on the significant increase in cellular apoE by GW3965 (**Fig. 3.8C**). When analysed by a Student t-test, the attenuating effect of RU486 on cellular apoE levels nearly reached significance with progesterone ($p=0.06$) and lynestrenol ($p=0.06$) (**Fig. 3.8C**). Like GW3965, lynestrenol and progesterone significantly increased cellular ABCA1 protein (**Fig. 3.8D**, $p<0.0001$ for GW3965 and lynestrenol, $p<0.05$ for progesterone). Unlike apoE, however, ABCA1 protein levels were unaffected by RU486 (**Fig. 3.8D**, $p>0.05$), indicating that the PR does not participate in regulating ABCA1 expression.

Consistent with the protein data, RU486 showed no significant effect on apoE mRNA levels in vehicle control or in GW3965-treated cells but blocked the ability of progesterone and lynestrenol to increase apoE mRNA levels above baseline (**Fig. 3.8E**). By Student t-test, the decrease of apoE mRNA level in the presence of RU486 for both progesterone ($p=0.07$) and lynestrenol ($p=0.07$) treatment again nearly reached statistical significance (**Fig. 3.8E**). Also consistent with the protein data, ABCA1 mRNA levels upon treatment with progesterone, lynestrenol or GW3965 was not affected by RU486 (**Fig. 3.8F**, $p>0.05$). Together, these data suggest that lynestrenol and progesterone induce ABCA1 expression solely via an LXR-dependent mechanism, whereas the mechanism by which lynestrenol and progesterone stimulate apoE secretion also involves the PR.

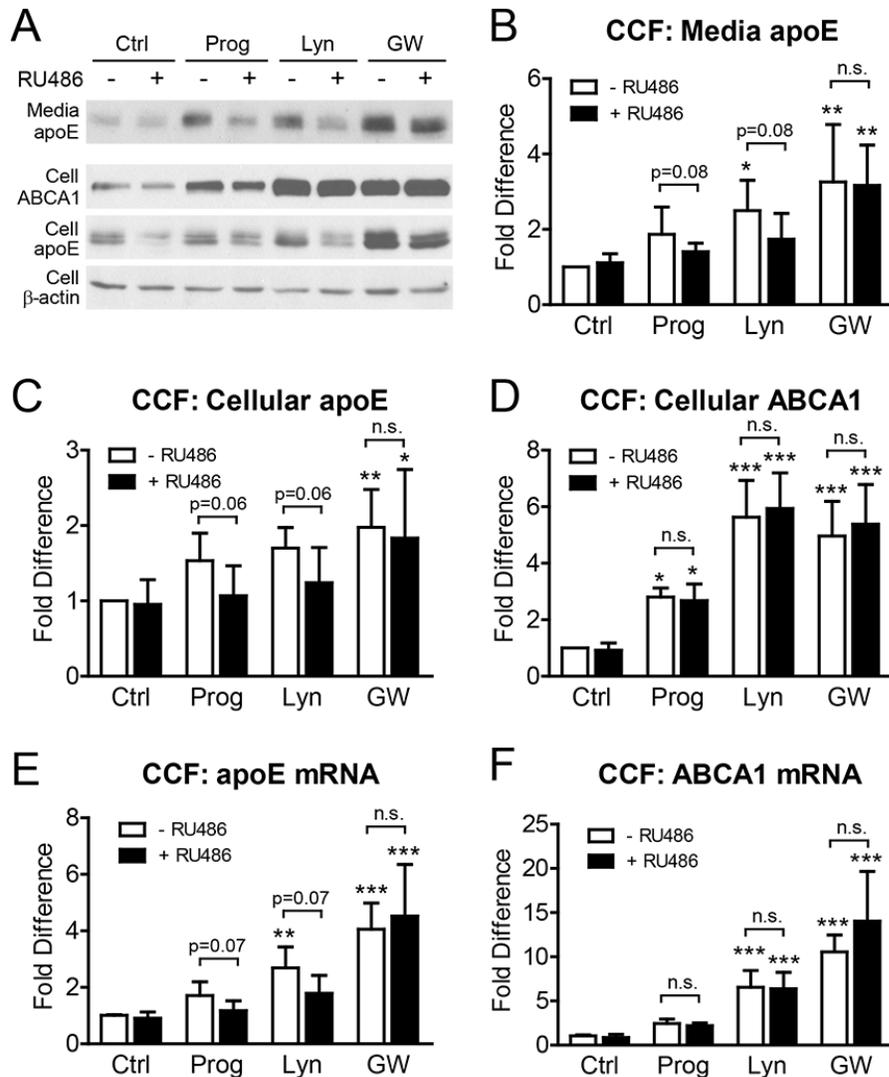


Figure 3.8: Upregulation of apoE by lynestrenol in CCF-STTG1 cells is partially progesterone receptor dependent.

CCF-STTG1 cells in 12-well plates were pre-treated with 5 μ M of RU486 or DMSO alone for 1h, followed by treatment of DMSO alone (Ctrl), 1 μ M of GW3965 (GW), 10 μ M of lynestrenol (Lyn) and progesterone (Prog) with or without 5 μ M RU486 for another 24h. Western blotting (A) was used to measure media apoE (B), cellular protein levels of apoE (C) and ABCA1 (D) in whole cell lysates. Band intensity was quantitated by densitometry and expressed as fold change relative to the DMSO control without RU486 co-treatment. Real-time quantitative PCR was used to measure apoE (E) and ABCA1 (F) mRNA levels in whole cell lysates. Data represent mean and SD of fold differences from four independent experiments for B, six experiments for C and D, five experiments for E and F. Drug effect of progestins and GW3965 was analysed by two-way ANOVA with a Tukey's post test. Effect of RU486 was analysed by two-way ANOVA with a Sidak's column comparison. * represents $p < 0.05$, ** represents $p < 0.01$, and *** represents $p < 0.0001$. P-values above the brackets are calculated from Student t-test.

3.3.8 Lynestrenol increases ABCA1 levels in two other astrocyte model systems.

To determine the effect of progestins on astrocytes other than human astrocytoma CCF-STTG1 cells, we then assessed primary murine mixed glial cells consisting of approximately 99% astrocytes and 1% microglia as well as immortalized astrocytes derived from human apoE3 or apoE4 knock-in mice (Morikawa et al., 2005). Because we have previously shown that primary murine glia have a slower accumulation of apoE in media (J. Fan et al., 2011), drug exposure time was extended to 72h for primary glia as opposed to 24h with CCF-STTG1 cells and immortalized apoE3- and apoE4- expressing astrocytes. In addition, the doses of progesterone and lynestrenol were decreased from 10 μ M to 1 μ M when treating immortalized apoE3- and apoE4-expressing astrocytes due to cellular toxicity of higher doses of steroid hormones in these cells.

Compared to human CCF-STTG1 cells, immunoblotting experiments demonstrated that both primary murine glia and immortalized murine astrocytes from apoE3 or apoE4 targeted replacement mice had a less robust response to all drug treatments, possibly suggesting a species difference compared to apoE regulation in human CCF-STTG1 cells. Specifically, primary murine glia showed no increase in apoE secretion upon treatment with progestins or GW3965 (See Appendix A supplemental Fig. 2), whereas both apoE3- and apoE4-expressing astrocytes showed a trend toward increased apoE secretion in response to GW3965 treatment (See Appendix A supplemental Fig. 3). No significant changes in cellular apoE protein or mRNA were observed in either primary glia or in apoE3- or apoE4-expressing astrocytes. The kinetics of apoE secretion and toxicity thresholds may account for the differences observed in these cellular models compared to CCF-STTG1 cells. Nevertheless, lynestrenol led to a 2.07-fold

significant increase in ABCA1 protein levels over baseline in primary murine glia (See Appendix A supplemental Fig. 2), a 3.81-fold significant increase in apoE3-expressing astrocytes and a 3.88-fold trend increase in apoE4-expressing astrocytes (See Appendix A supplemental Fig 3) in the absence of RU486. Progesterone also significantly increased ABCA1 protein levels by 1.74-fold over baseline in primary murine glial cells, and induced a trend increase of ABCA1 in both apoE3-expressing (2.23-fold) and apoE4-expressing astrocytes (2.56-fold). RU486 did not affect ABCA1 protein expression in either primary murine glia or in apoE3- and apoE4-expressing astrocytes, again suggesting that the PR does not regulate ABCA1 expression in glia. ABCA1 mRNA analysis on primary murine glia also indicated a comparable upregulation induced by lynestrenol and GW3965 and confirmed that RU486 does not affect LXR-mediated stimulation of ABCA1 in glia (See Appendix A supplemental Fig 2).

Together, these data suggest that primary murine glia and immortalized murine astrocytes expressing human apoE3 and apoE4 have a less robust response to progestins and GW3965 compared to human CCF-STTG1 astrocytoma cells. However, lynestrenol still exhibits LXR-agonist behavior as it upregulates ABCA1 expression independent of the PR in both primary murine glia and immortalized apoE3- and apoE4-expressing murine astrocytes. Finally, we observed no marked difference in response of apoE3- versus apoE4-expressing astrocytes to any of the treatments, suggesting that these effects are likely to be independent of apoE isoform.

3.4 Discussion

In a screen for compounds that increase apoE secretion from human astrocytoma cells, we identified the synthetic progestin lynestrenol as well as progesterone among the hits. Lynestrenol is marketed as a component of several oral contraceptives (OC) in Europe and Asia and is also prescribed for menstrual disorders including endometriosis. This orally available progestin is reported to have strong progestagenic activity but low estrogenic, androgenic and anabolic effects (Schindler et al., 2008). Our study demonstrates that lynestrenol and progesterone differ with respect to their ability to serve as LXR agonists. Specifically, we show that lynestrenol exhibits LXR agonist activity in glial cells roughly comparable to the known LXR agonist GW3965 with respect to ABCA1 induction, whereas progesterone has weak LXR activity and the progesterone metabolite allopregnanolone has none. We also show that ABCA1 expression is LXR- but not PR-dependent, and, in human CCF-STTG1 cells, that apoE can be regulated by LXR as well as PR-dependent pathways.

Epidemiological data from OC users in the 1960s clearly associated the high dose formulations used in that era with increased risk of ischemic stroke, myocardial infarction and pulmonary embolism in healthy young women, particularly for smokers (G. Fazio et al., 2010; Fotherby, 1989). Several studies of older high dose OC formulations including those containing lynestrenol typically showed detrimental effects on cardiovascular risk factors including reduced HDL levels and increased LDL and TG levels (reviewed in Fotherby (1989)). However, it is important to note that these changes in lipid levels are reported to be due to effects on hepatic lipase, which is generally stimulated by estrogens and inhibited by progestins including lynestrenol (Fotherby, 1989). As a marketed drug, the reported adverse effects of lynestrenol do not include hepatic

steatosis, and several studies report either no change or decreased serum TG levels (Graff-Iversen & Tonstad, 2002; Huovinen et al., 1990; Radberg et al., 1982; Teichmann et al., 1988). These clinical studies, along with our demonstration that the LXR agonist activity of lynestrenol is modest with respect to SREBP-1c in HepG2 cells, suggests that lynestrenol may have a favorable safety profile compared to more recently developed synthetic LXR agonists such as TO901317 and GW3865. Based on the known beneficial effects of LXR agonists in murine AD models, it is possible that that lynestrenol may therefore combine the neuroprotective effects of progesterone and the beneficial effects of an LXR agonist in a single compound with a potentially acceptable safety profile.

Many oxysterols, such as 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol and 27-hydroxycholesterol, are natural LXR ligands (Janowski et al., 1996; Lehmann et al., 1997). 24(S)-hydroxycholesterol induces apoE secretion, ABCA1 expression and apoE-mediated cholesterol efflux in CCF-STTG1 cells via an LXR-controlled pathway (Abildayeva et al., 2006). Lynestrenol, progesterone and other progestins have structural similarity to these oxysterols. However, other progestins, including medroxyprogesterone, D-norgestrel, chlormadine acetate, cyproterone acetate and 19-norethindrone, had no significant effect on apoE secretion. Although not exhaustive, these results suggest that there is considerable selectivity in the ability of natural and synthetic progestins to modulate apoE levels. There may also be particular features of steroid hormone structure that modulate LXR activation, which is separable and distinct from their endocrine activities. Our study provides new insights about the structure-activity relationship for LXR-agonists.

The neuroprotective role of progesterone is of considerable interest, particularly as a potential treatment for traumatic brain injury (TBI) as it promotes neuronal survival, attenuates oxidative damage and edema, exerts anti-inflammatory effects and facilitates myelination of regenerating neurons (Sayeed & Stein, 2009; Stein, 2001; Stein & Hoffman, 2003). The efficacy of progesterone as a treatment for TBI was recently reported in the ProTECT study, a randomized, double-blind, placebo-controlled phase II clinical trial that showed that moderate brain injury survivors who received progesterone were more likely to show improvement than those receiving placebo (Wright et al., 2007). Progesterone is thought to exert CNS effects via three principle mechanisms: activation of signaling cascades, modulation of neurotransmitter systems and regulation of gene expression (Brinton et al., 2008). In addition to the two major isoforms of the classic nuclear progesterone receptor, PRA and PRB, recent studies reveal a novel progesterone-binding membrane protein (mPR) that has characteristics of a G protein-coupled receptor that activates the mitogen-activated protein kinase (MAPK) pathway and extracellular signal regulated kinase (ERK) pathways (Zhu et al., 2003a; Zhu et al., 2003b). Some of the neuroprotective effects of progesterone may therefore be via the MAPK/ERK pathways that modulate cellular differentiation, proliferation, survival and cell death (Nilsen & Brinton, 2002, 2003; Singh, 2001).

Our data suggest that PR activity contributes to apoE but not ABCA1 expression, and progesterone's effect on apoE modulation may primarily be at the post-translational level. Duan et al. (2000) showed that progesterone increases apoE secretion from macrophages by acting on the C-terminal lipid-binding domain of apoE to block its intracellular degradation. We observed that progesterone decreases cellular LDLR receptor levels, suggesting that a decrease in

uptake/recycling of apoE may contribute to the elevated steady state level of secreted apoE. Considerable evidence shows that synaptogenesis, efficient neurite outgrowth, and regrowth in response to injury rely on lipid transport by apoE (H. Hayashi et al., 2004; Holtzman et al., 1995; Mauch et al., 2001; Nathan et al., 1994). We therefore hypothesize that some of beneficial effects of progesterone in the CNS in response to brain injury such as TBI may partially arise from its ability to modulate apoE production and lipidation.

Although estrogens are also reported to be neuroprotective (Brinton et al., 2008), we found that only 17 β -estradiol stimulated apoE secretion very modestly whereas estrone, 17 α -estradiol, estriol, β -estradiol 17-valerate, β -estradiol 3-benzoate, Raloxifene hydrochloride, Tibalone and Premarin showed no significant effect. Nevertheless, in vivo studies demonstrated that treating ovariectomized female mice with exogenous 17 β -estradiol increased apoE levels in brain tissue (Levin-Allerhand et al., 2001; McAsey et al., 2006; Struble et al., 2003). Nathan et al. (2004) also showed that 17 β -estradiol was able to increase apoE secretion and prompt apoE-mediated neurite outgrowth in primary murine mixed CNS cell culture (70% neurons, 15% astroglia and 15% microglia). Intriguingly, Stone et al. (1997) showed that 17 β -estradiol treatment had no effect on apoE mRNA levels in monotypic primary cultures of either astrocytes or microglia, yet mixed glial cultures exposed to 17 β -estradiol showed increased apoE mRNA and protein. In parallel, Premarin (i.e. conjugated equine estrogens, CEE, containing >50% estrone, 15-25% equilin and equilenin) also induced apoE expression in mixed glia (Rozovsky et al., 2002) but have no effect in monotypic astrocytoma cells. Together, these data suggest that estrogen may require heterotypic cellular interactions to modulate apoE.

Limitations of our study include challenges related to in vitro cellular model systems. Human CCF-STTG1 astrocytoma cells were used for the majority of experiments due to their ease of use and well-characterized robust response to LXR stimulation. However, as a naturally transformed cell line, CCF-STTG1 cells may have altered apoE regulatory pathways that enhance tumor growth. In contrast to CCF-STTG1 cells, lynestrenol and GW3965 failed to significantly elevate apoE secretion in primary murine mixed glia or immortalized murine astrocytes derived from human apoE3 and apoE4 knock-in mice under our experimental conditions, despite a clear effect on ABCA1. These differences suggest a possible species difference in how lynestrenol or GW3965 regulates glial apoE production, as the endogenous murine apoE promoter drives apoE expression in both primary murine glia and in the apoE3 and apoE4 expressing immortalized astrocytes (Morikawa et al., 2005). In addition, the three cellular models used have different toxicity profiles in response to drug treatment and different tolerances to serum-free growth conditions, which may also partially account for the different magnitude of the responses observed in the cell types studied. Despite these limitations, the LXR agonist activity of lynestrenol on ABCA1 expression is consistent among the cell types tested.

Chapter 4: Conclusions

As discussed in Chapter 1, several lines of evidence suggest that altered CNS lipid metabolism may contribute to AD pathogenesis. ApoE is a key player in CNS lipoprotein metabolism and the most important genetic risk factor for late-onset AD. Previous work from our laboratory showed that the cholesterol transporter ABCA1 is a key regulator of apoE levels and lipidation status in the CNS and that deficiency of ABCA1 increases amyloid burden in AD mouse models. During the course of this thesis work, one paper published by the Landreth group (Jiang et al. 2008) discovered that ABCA1-mediated lipidation of apoE may facilitate the enzymatic degradation and clearance of A β peptides, offering a potential mechanism for novel therapeutic strategies for AD and other neurodegenerative conditions that involves A β pathology. Developing such therapeutic approaches requires a more thorough understanding of the biochemical nature of the nascent glia-derived apoE-containing lipoprotein particles and of lipoprotein remodeling in CNS in general. Meanwhile, identifying new apoE modulators that may regulate the secretion, lipidation and/or recycling of CNS apoE not only will broaden our basic knowledge on apoE biology but may also be of great therapeutic value given the current limitations of existing small molecule LXR agonists. Therefore, the central objective of this thesis is to study how apoE is remodeled and regulated in CNS via secretion, lipidation and recycling. This thesis work contributes to our understanding of brain lipoprotein and lipid metabolism from two aspects: the first half of this thesis provides novel findings in fundamental apoE biology describing both ABCA1-dependent and independent pathways for apoE lipoprotein particle secretion, while the second half of the thesis consists of translational research that explores the mechanisms of action of several apoE modulators that may have potential therapeutic use. While part of the data

generated in this thesis work has affirmatively answered the main hypothesis of this thesis, namely that ABCA1 plays an essential role in regulating glial apoE secretion, lipidation and recycling, this thesis also demonstrates ABCA1-independent pathways involved in the secretion and remodeling of CNS apoE-containing lipoproteins. Moreover, this thesis work has identified two steroidal hormones, progesterone and lynestrenol, as potent apoE modulators that not only induce apoE secretion levels but also enhance ABCA1 functioning, which may be worthwhile for future evaluation for therapeutic use.

4.1 Chapter 2: conclusions and future directions

In Chapter 2 of this thesis, I show that while ABCA1 facilitates the rapid secretion and lipidation of glia-secreted apoE to form a set of particles of different sizes (7.5-17 nm), there also exists an ABCA1-independent pathway that promotes the secretion of a poorly lipidated 8.1 nm particle subspecies from both WT and ABCA1^{-/-} glial cells. This small particle subspecies may arise through apoE recycling and is recycled primarily through LDLR in glia.

Similar to plasma HDL, which is a heterogeneous mixture of particle subclasses with distinct structural and functional difference, CNS apoE-containing lipoprotein particles generated by WT glia cells also exhibits a set of particle subspecies with difference sizes. The study in Chapter 2 provides evidence showing the structural and physiological differences in these subspecies. The study demonstrates that the particle diameters are modulated over a 6-72 h time course, suggesting an extensive remodeling of apoE species in the presence of astrocytes. Interestingly, both WT and ABCA1^{-/-} glia secrete an 8.1 nm apoE particle, which is shown to be structurally and functionally distinct from the other species. This 8.1 nm species is lipid-poor but can be

rapidly lipidated to form the full repertoire of particles normally seen in WT conditioned media if cells express functional ABCA1. Due to its delayed appearance over the time course, we hypothesized that this particle represents a pool of recycled apoE. Indeed, the 8.1 nm particle species is barely detectable in LDLR^{-/-} glia conditioned media. Also, by blocking apoE receptor function using RAP, the appearance of this 8.1 nm species is almost abolished in ABCA1^{-/-} glia conditioned media. We then show that apoE recycling, primarily through LDLR in glial cultures, contributes to the steady state of secreted apoE levels in media. LDLR expression is down-regulated to 25% of normal levels in ABCA1^{-/-} glia due to the lipid accumulation. Interestingly, while the overall apoE levels in brain tissues of ABCA1^{-/-} mice are significantly reduced, apoE secretion is not impaired in glial culture after an extended conditioning period. However, when we knock out the remaining 25% of LDLR in ABCA1^{-/-} glia, apoE secretion is greatly reduced to approximately 10% of WT levels, suggesting that LDLR is critical to maintain the ABCA1-independent secretion of apoE. HDL and apoA-I have been shown to stimulate apoE recycling in peripheral cells. We then demonstrate that in glial cells, apoA-I stimulates the secretion of the 8.1 nm apoE particles in a dose-dependent manner and induces cellular LDLR expression, further supporting the hypothesis that this 8.1 nm species raised from LDLR-mediated apoE recycling.

We have shown that apoE-containing lipoproteins secreted by glial cells is not a single entity but a heterogeneous pool of different particle subspecies generated by both ABCA1-dependent and independent pathways. The next important question for future studies is whether the various apoE lipoprotein subspecies vary in their interactions with A β and how such interactions may or may not affect CNS A β clearance via either degradation or transport out of the brain. Another

important question is how human apoE isoforms differ in terms of their subspecies distribution and function. For example, Michikawa et al. (2000) reported that exogenously added recombinant human apoE isoforms promoted the cholesterol efflux from primary cultures of rodent neurons and astrocytes in an isoform-specific manner. Yet, it is unknown whether different apoE isoforms have particle subspecies that exhibit distinct characteristics that may lead to difference in their ability to promote cholesterol efflux and/or other functions. Ideally, astrocyte-derived conditioned media containing each of the isoforms should be used to test this hypothesis, as it corresponds to a more biologically relevant lipoprotein preparation, both in the conformation and lipidation of the secreted apolipoproteins than recombinant apolipoproteins. Not only that, it would be more ideal if each individual subclass of glia-derived apoE particles can be isolated and purified to a large quantity for downstream analyses such as cholesterol efflux assay, A β interaction/degradation assays and effects on neuronal repair and synaptogenesis.

As mentioned in Chapter 1, Section 1.4.3, Fitz et al (2012) has demonstrated that loss of one gene copy of ABCA1 could worsen the cognitive performance, amyloid burden and CNS A β clearance in human apoE4-expressing AD mice. Interestingly, in my study I observed that the pattern of apoE-containing particle size distribution found in the conditioned media collected from ABCA1^{+/-} murine glia was no different from the wild-type glia. My preliminary RNA interference (RNAi) study that used siRNA to knockdown ABCA1 in primary murine glia also showed that the size pattern of glia-derived apoE particles was not altered even with 80% reduction of ABCA1 protein level, suggesting that the remaining 20% of ABCA1 protein was sufficient to generate full repertoire of apoE particles as found in the wild-type media. However,

detailed analyses on the lipid composition and other biochemical and structural features of such particles are needed to fully understand how loss of one gene copy of ABCA1 may result in a different apoE-lipoprotein metabolism. In particular, why is apoE4 specifically affected by ABCA1 hemizyosity? It would be very interesting to examine the features of the CNS lipoproteins derived from the animals studied in Fitz study. Continuing research on characterizing CNS-derived apoE-containing lipoprotein and apoE function in the healthy and diseased brain is needed to help us understand and overcome the detrimental aspects of apoE4 for both acute and chronic neurological conditions.

4.2 Chapter 3: conclusions and future directions

In Chapter 3 of this thesis, two hormonal modulators for apoE secretion were identified and studied for their action of mechanisms. Although current synthetic LXR agonists including TO901317 and GW3965 have shown promising beneficial effects in several AD mouse models, including one study presented in Appendix B.1 to which the thesis author made contributions, these synthetic compounds also activate liver SREBP-1c and fatty acid synthase pathways which leads to undesired side effects such as hepatic steatosis and hypertriglyceridemia in species such as humans that express CETP. This becomes a significant caveat for current LXR agonists and currently precludes their translation into human clinical trials. Therefore, it is important to search for alternative methods to enhance apoE function in the brain. In Chapter 3 we report that a synthetic progestin, lynestrenol, significantly enhances apoE secretion from CCF-STTG1 human astrocytoma cells while natural progesterone also moderately stimulates apoE secretion from this cell model. Interestingly, cellular ABCA1 levels are also effectively induced by both lynestrenol and progesterone, an effect that is LXR-dependent. Our data suggests that lynestrenol has a

substantial LXR agonist activity that increases ABCA1 expression and promotes cholesterol efflux, while progesterone has relatively weak LXR activity. Moreover, when treating with a high dose of progesterone (10 μ M), astrocytoma cells show a trend of decreased LDLR levels, implicating progesterone-induced reduction of apoE uptake may possibly also contribute to the elevated steady state apoE levels in the media pool. Another possible mechanism that these two hormonal compounds exert their effects on apoE secretion is via the progesterone receptor pathway, as pharmacological inhibition on progesterone receptor activity has attenuated the ability of lynestrenol and progesterone to increase apoE expression. On the other hand, induction of ABCA1 expression by the two hormones is PR-independent. Together, these results offer new insights into how specific reproductive steroid hormones may influence glial lipid homeostasis through multiple pathways.

Results from Chapter 3 indicate a possible involvement of progesterone receptor in regulating apoE expression and secretion. The question of whether PR may have direct transcriptional control over apoE expression or it involves other indirect pathways requires further investigation. Particularly, bioinformatics analysis on whether apoE promoter region contains any PR binding site will be informative. It will be also of interest to use bioinformatics analysis to elucidate the species differences in the apoE promoter region. In chapter 3, we have evaluated the progesterone/progestin effects on two other glia models in addition to human CCF-STTG1 astrocytoma cells. However, lynestrenol and GW3965 both failed to significantly elevate apoE secretion in primary murine mixed glia or immortalized murine astrocytes derived from human apoE3 and apoE4 knock-in mice despite a clear effect on ABCA1 expression. These observations suggest a possible species difference in glial apoE regulation, as the endogenous

murine apoE promoter drives apoE expression in these two murine glial models. This also raises the question whether mouse cell models or in vivo mouse models are valid or useful for drug discovery research targeting apoE. This may be an important consideration for studies using the targeted replacement apoE mice, as the human apoE genes are driven by the murine apoE promoter.

In Chapter 3, it is shown that progesterone's effect on apoE modulation can be at the post-translation level as we observed a trend toward reduced cellular LDLR receptor levels by progesterone treatment. Recent studies from the Tontonoz group (reviewed in Zhang et al. (2012)) show that degradation of LDLR can be mediated by an ubiquitin ligase now named as IDOL, inducible degrader of the LDLR. Interestingly, in macrophages and hepatocytes, IDOL expression is controlled at transcriptional level by LXR. The Tontonoz group first observed a marked reduction of cellular LDLR protein levels upon treating macrophages and hepatocytes with GW3965 and TO901317 for only 4 h. However LDLR mRNA levels remained unchanged, but rather LXR activation resulted in a rapid elimination of LDLR protein from the cell surface (Zelcer et al., 2009). Through transcription profiling, the researchers then identified IDOL as the mediator of the effects of LXR on the LDLR, as the IDOL gene promoter is a direct target for binding and regulation by LXR/RXR heterodimers. The unanswered question is whether this LXR-IDOL-LDLR pathway is active in CNS cells as well. Interestingly, in my study presented in Chapter 3, 24 h-treatment of 1 μ M GW3965 did not decrease LDLR protein levels in CCF-STTG1 astrocytoma cells. Yet in a preliminary study, we started to see a trend decrease of LDLR by 1 μ M GW3965 treatment after 48 h, suggesting the effect of this LXR agonist on LDLR may be much slower or less robust than that found in the peripheral cells. Further detailed analysis on

IDOL expression in CNS cell types under LXR agonist treatment is needed to elucidate the significance of this LXR-IDOL-LDLR pathway in the CNS. Since progesterone also leads to a reduction of LDLR in astrocytoma cells, future studies should investigate whether IDOL is involved in this progesterone-induced effect. If progesterone indeed regulates IDOL expression, the next interesting question will be whether progesterone exerts this regulatory effect through LXR pathway or/and PR pathway.

The study in Chapter 3 also suggests that the possible role of female reproductive hormones in AD should be re-visited and further explored. Estrogen and progesterone have long been implicated in AD as women have a greater risk of dementia than men in their later years (Farrer et al., 1997). Previous studies report that women carrying at least one APOE ϵ 4 allele are at increased risk of developing AD compared to men of the same apoE genotype (Breitner et al., 1999; Bretsky et al., 1999; Payami et al., 1996). Various observational and retrospective studies suggest a protective role of exogenous estrogen in decreasing the likelihood of late-life dementia (Baldereschi et al., 1998; Kawas et al., 1997; Paganini-Hill & Henderson, 1994; Tang et al., 1996; Yaffe et al., 2000; Zandi et al., 2002). However, the Woman's Health Initiative Study (WHI) showed that neither estrogen alone (Premarin) nor estrogen plus progestin (Premarin plus medroxyprogesterone acetate (MPA)) therapy reduced dementia or mild cognitive impairment incidence but rather resulted in increased risks for both endpoints in postmenopausal women aged 65 or older (Shumaker et al., 2003). In parallel, despite numerous retrospective studies demonstrating cerebrovascular and cardiovascular benefits of HRT among postmenopausal women (Maclaran & Stevenson, 2012), the WHI study reported an increased risk for stroke and cardiovascular disease (Manson et al., 2003; Wassertheil-Smoller et al., 2003). One explanation

for the detrimental effects of HRT in the WHI study may be in the timing of the HRT initiation. The vast majority of women in the WHI study were on average 12 years postmenopausal prior to the initiation of HRT, whereas in observational studies that report beneficial effects of HRT, the majority of subjects initiated hormone replacement in their perimenopausal period. Indeed, a recent early-intervention study demonstrated a beneficial effect of HRT on AD. Compared to women not using HRT, women who initiated therapy within 5 years of menopause had a 30% lower incidence of AD (Shao et al., 2012), providing further evidence that there exists a critical window of initiation timing for HRT to exert its beneficial effects.

Another crucial question raised by the WHI study is whether synthetic progestogenic and estrogenic compounds may have considerably different effects compared to human bioidentical progesterone and estrogens. The estrogen and progestin used in the majority of HRT studies including the WHI are CEE and MPA. Fundamental differences of biological effects can exist between CEE versus estradiol and between MPA versus natural progesterone. For example, unlike natural progesterone, MPA does not bind to mPR to activate MAPK/ERK signaling (Nilsen & Brinton, 2003). Our study also showed that MPA does not affect apoE secretion. Therefore, while MPA has similar progestagenic activity to progesterone, it may not be able to exert same neuroprotective effect as progesterone does.

Furthermore, very little is known about how apoE genotype affects the response to different formulations of HRT. As apoE4 is dysfunctional relative to apoE3 and apoE2 and the net levels of apoE4 in brain tissue of human AD patients are reduced (Mahley et al., 2006), compounds that elevate apoE mass and activity may be of potential therapeutic interest for AD. On the other

hand, hemizyosity of apoE2/3/4 decreases amyloid burden in AD mice, and complete deficiency of apoE nearly eliminates the formation of amyloid plaques. Thus, it is becoming increasingly important to understand the factors that regulate apoE production and function in the CNS. The vast epidemiological data on the increased risk of AD in post-menopausal women and the neuroprotective effect of female reproductive hormones provides a clear rationale for future research in this field, particularly as the potential interactions of apoE genotype in women on HRT are largely unexplored.

4.3 Limitations and caveats

The major limitation of this thesis work is that the models studied are solely *in vitro*, being either cultured primary cells or immortalized cell lines. The extraordinary complexity of living organisms is a great obstacle for scientists to identify individual components and explore their biological functions. Therefore, the major advantage of *in vitro* work is that it provides an enormous level of simplification of the system to study so that researchers can focus on a small number of components. However, while *in vitro* models provide the simplicity needed to test very specific hypotheses, the limitations of *in vitro* studies should not be overlooked. Caution must be taken when we extrapolate from the results of *in vitro* work back to the biology of the intact organism, as over-interpretation of the results can lead to erroneous conclusions at the systematic level. For example, in my study presented in Chapter 3, estrogen treatment on human astrocytoma cells showed negligible effect on apoE secretion; however, other studies showed that 17 β -estradiol was able to significantly increase apoE secretion in primary mouse mixed glial/neuronal culture (Nathan et al., 2004) and in rat mixed glial culture (Stone et al., 1997). These data together suggest that estrogen may require heterotypic cellular interactions to

modulate apoE. Advancing techniques in heterotypic cell culturing and in vitro organ culturing, which may retain more systematic information at a higher level than monotypic culture does, may provide us better tools and models to conduct in vitro studies that are more physiologically meaningful.

Primary cell culture is often preferred over immortalized cell line culture for its physiological relevance, but primary cell culturing is limited in terms of the amount of cells generated and the growth time required is often long (usually 2-3 week long for glial culture). Although easy to maintain and fast to grow, the tumorous immortalized cell lines, such as the CCF-STTG1 astrocytoma cells used in this thesis work, often have altered cellular metabolism which could become confounding factors that make the interpretation of the data not easy. For example, unlike the self-sustaining primary murine glial cells that can survive in serum-free media for many days without any observable morphological change, CCF-STTG1 cells cannot tolerate serum-free environment for more than 48h as observed in my study. Whether and how this feature of CCF-STTG1 cells may affect their apoE metabolism is unclear. Immortalizing primary cells with viral vectors, such as SV40, is another common practice to generate large amount of cells in a short time for high throughput experiments. For example, the human apoE3 and apoE4 expressing astrocytes used in the study in Chapter 3 were derived from mouse primary astrocytes that were immortalized by SV40 (Appendix A Figure A.3). However, the dramatic alteration in cell metabolism upon immortalization may have unpredictable effects on the particular pathways of interest. I observed that the immortalized human apoE-expressing murine astrocytes exhibit a different toxicity profile in response to drug treatment than primary murine astrocytes that express endogenous murine apoE. While 10 μ l of lynestrenol was not toxic to primary murine

astrocytes, this same dose of lynestrenol almost killed all the immortalized astrocytes within 24h of drug treatment, thus the dose of 1 μ l of lynestrenol had to be used for later experiments. Whether or not this difference in toxicity profile is due to immortalization or the presence of apoE from different species is entirely unclear, although it is unlikely due to the later, since 10 μ l of lynestrenol was not toxic for human CCF-STTG1 cells. Nevertheless, these observations suggest that interpretation of in vitro data is complicated and needs to be made with great caution. Species-specificity is another problem for in vitro studies (and for in vivo as well), which has been discussed in the above Section 4.2. Human primary cells would be a more ideal in vitro model to study how different human apoE isoforms are regulated, particularly at the transcriptional level. However, it is very difficult to obtain primary culture of human CNS cells. Therefore, despite the above discussed limitations, cell lines and primary murine cell culture remain to be workable models for generating initial data that may provide insights to the directions for further in vivo and/or clinical studies.

Seeing the limitation of in vitro approach, in addition to my own in vitro studies, I have also participated and made contributions to several in vivo studies that are closely related to the topic of this thesis work. In Appendix B, I summarized three published co-author papers that are closely relevant to the main hypothesis of this thesis. The first two studies focus on evaluating the potential therapeutic values of the LXR agonist GW3965 in treating AD and traumatic brain injury (TBI), and testing whether ABCA1 or apoE is required for the compound to exert its beneficial effects. The study by Donkin et al. (2010) is the first to specifically demonstrate ABCA1 as a key component of LXR beneficial response (Appendix B.1). ABCA1 is required for GW3965 to show beneficial effects in terms of improving cognitive functions and reducing total

amyloid burden in AD mice. Without ABCA1, apoE may not carry out its normal beneficial functions in CNS. Yet, the ABCA1^{-/-} mouse model used in this study only presents an extreme scenario of the “loss of function” of ABCA1. For the purpose of drug discovery, the more important question will be whether a drug must modify both apoE and ABCA1 function to have a meaningful therapeutic effect. In other words, will an apoE modulator that solely regulates apoE levels without enhancing ABCA1 function (lipidation) be beneficial or not? This is an important question to answer when considering strategies for drug research. Further studies that validate whether specifically targeting ABCA1 is a viable therapeutic strategy are also needed.

The second in vivo study by Namjoshi et al. (2013) demonstrates that GW3965 improves recovery from TBI in a mouse model (Appendix B.2). Both apoE-dependent and apoE-independent pathways contribute to the beneficial effects of the LXR agonist on TBI. Loss of apoE eliminated the ability of GW3965 to restore cognitive performance and to promote axonal recovery, whereas apoE deficiency surprisingly did not hinder the ability of GW3965 to suppress elevated murine A β induced by injury. These observations suggest that in the apoE-deficient animals, LXR agonists may utilize other clearance routes, possibly transportation via the BBB, to reduce brain A β . Of note, amyloid deposition is almost completely diminished in apoE-deficient AD mice. Microdialysis studies suggest that the reduction of fibrillar A β deposits in apoE-deficient AD mice may in part be due to the improved A β transport across the BBB in the absence of apoE (Deane & et al., 2004), suggesting that apoE may compete with A β in pathways that allow egress of A β from the brain. Further studies will be needed to characterize the apoE-independent pathways that promote A β clearance after TBI.

The ABCA1^{-/-} glia used in the studies in Chapter 2 were derived from whole-body knockout mice, which were the same mice that were studied in Donkin 2010 study. Although it is less of a problem for an in vitro study, one caveat of this global knockout animal model for an in vivo study is that in mice lacking ABCA1 globally, the specific role of brain ABCA1 cannot be studied without concurrent changes in whole body cholesterol homeostasis. ABCA1^{-/-} mice have very low plasma HDL and apoA-I levels (Oram & Heinecke, 2005) which may diminish the pool of apoA-I available for transport into the CNS, which may in turn have an effect on CNS lipid metabolism. In the third co-author paper summarized in Appendix B, Karasinska et al. (2009) further investigated the role of ABCA1 in brain cholesterol homeostasis by using a mouse model with specific deletion of brain ABCA1 (Appendix B.3). Phenotypically, disturbances in motor activity and sensorimotor function are observed, possibly due to the changes in synaptic ultrastructure, including reduced synapse and synaptic vesicle numbers, which were observed in the mutant mice. The data from this study show that ABCA1 is a key regulator of brain cholesterol metabolism and that disturbance in cholesterol transport in the CNS are associated with structural and functional deficits in neurons. Intriguingly, we also found that loss of brain ABCA1 increases brain uptake of esterified cholesterol from plasma HDL. This study thus raises an important question as to whether cholesterol transport across the BBB is altered in neurodegenerative diseases. Also, CSF apoA-I levels were considerably elevated in the mutant animals, the implication of which is worthy of further study. Could this elevation of apoA-I be a compensatory response to the altered brain lipid homeostasis? Although apoA-I is not synthesized in brain cells (Ladu et al., 1998), several recent studies suggest that apoA-I may affect CNS function and impact the pathogenesis of AD. For example, deletion of apoA-I in an AD mouse model results in increased inflammation and impaired spatial learning (Lefterov et al.,

2010), whereas 2-fold overexpression of apoA-I protects AD mice from inflammation and age-associated learning and memory deficits (Lewis et al., 2010). These observations suggest that although apoA-I is not a major apolipoprotein in brain parenchyma, it may play a greater role in CNS functions than previously thought. Future investigations may be needed to evaluate apoA-I as another potential therapeutic target for treating neurodegenerative conditions.

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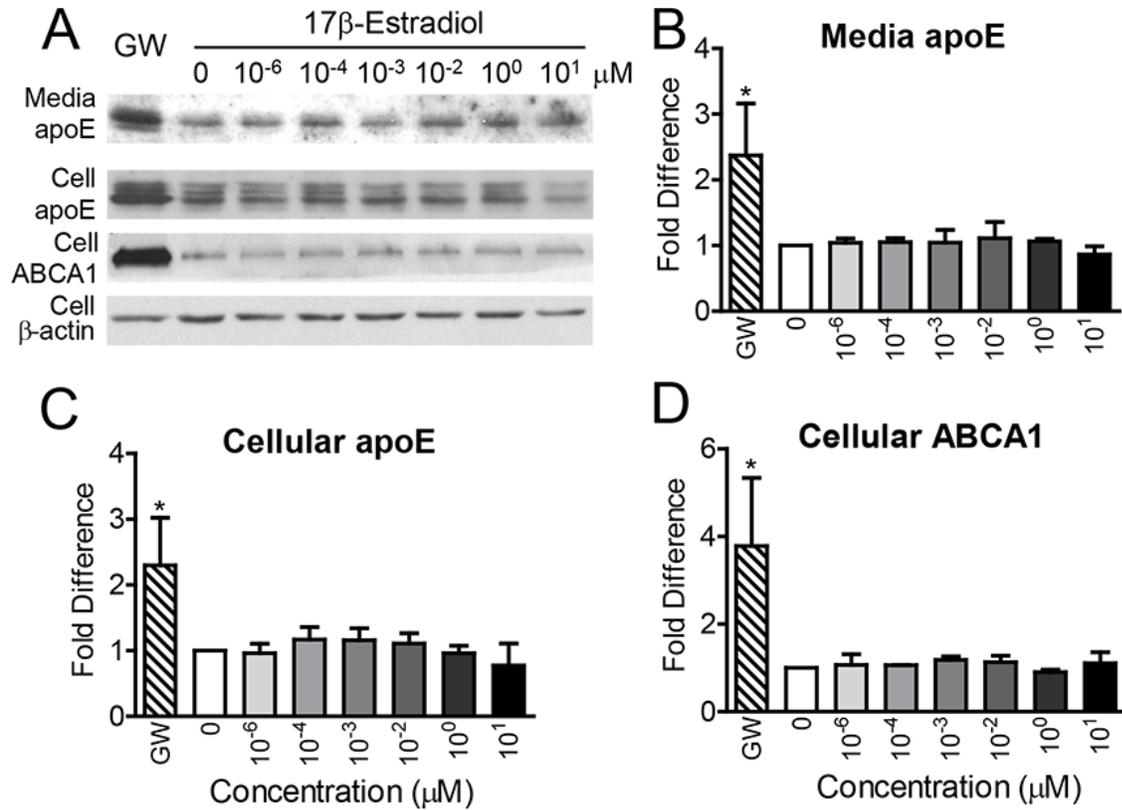
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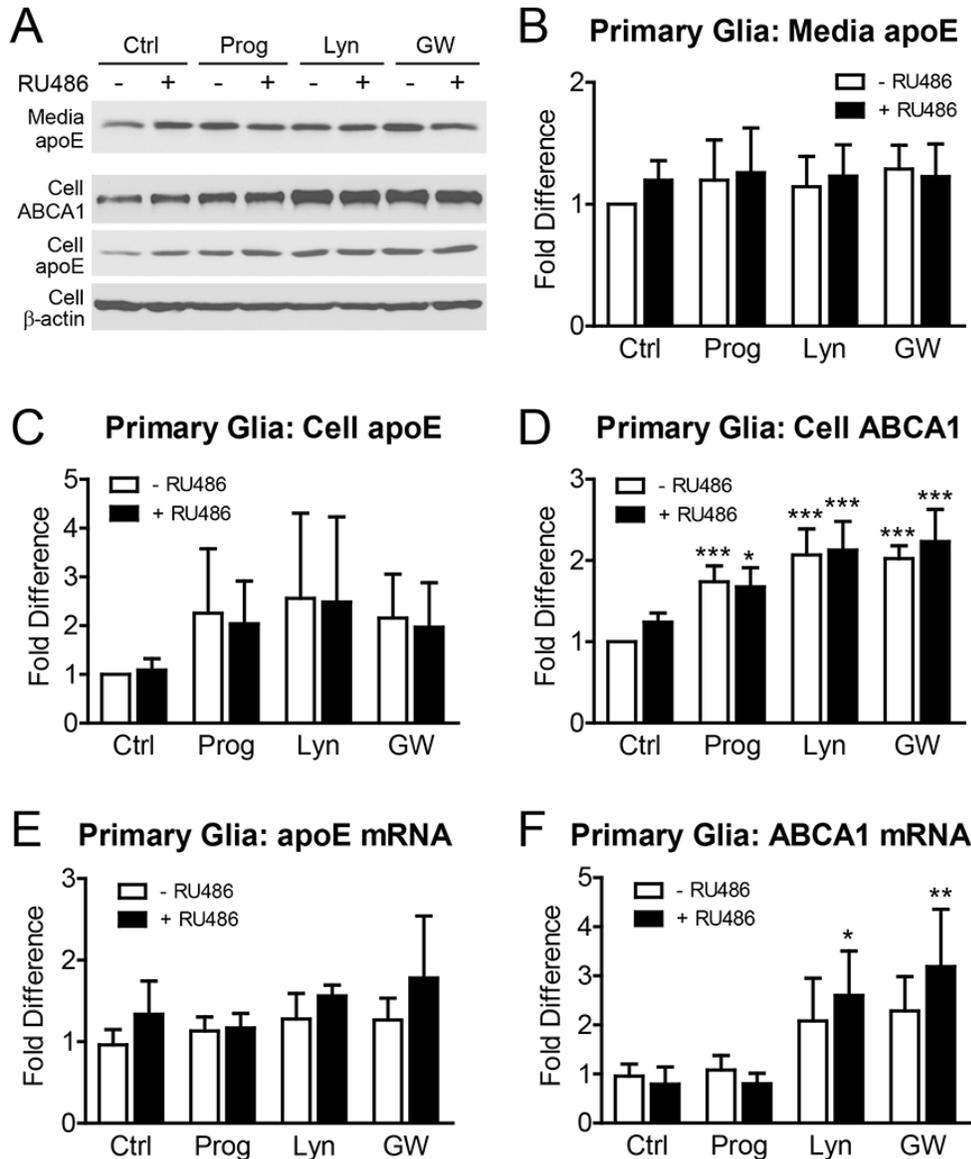
Appendices

Appendix A Supplemental figures for Chapter 3



Supplemental Figure A.1: 17 β -estradiol does not exhibit LXR agonist activity.

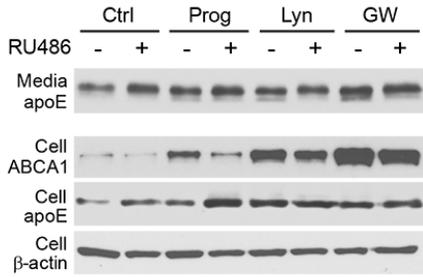
CCF-STTG1 cells were seeded in 12-well plates at 450K cells/well and treated with 17 β -estradiol at 1 pM, 100 pM, 1 nM, 10 nM, 1 μ M and 10 μ M for 24h, along with DMSO alone (0) as a negative control and 1 μ M of GW3965 (GW) as a positive control. (A) Representative Western blots of media apoE, cellular apoE, cellular ABCA1, and β -actin as a loading control. Band intensity was quantitated by densitometry and expressed as fold change relative to the DMSO control. Data represent mean and SD of fold differences of media apoE (B), cellular apoE (C) and cellular ABCA1 (D). Graphs illustrate two independent experiments, each in duplicate, analyzed by one-way ANOVA with a Tukey's post test. * represents $p < 0.05$.



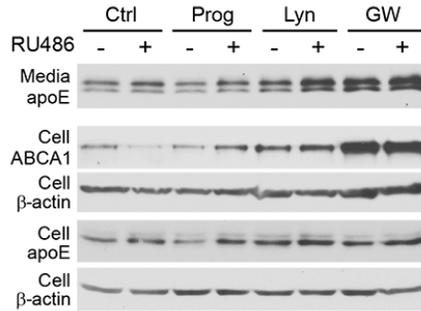
Supplemental Figure A.2: Effects of progesterone and lynestrenol in primary murine mixed glia.

Primary mixed glial cells were re-seeded in 12-well plates and pre-treated with 5 μ M of RU486 or DMSO alone for 1h, followed by treatment of DMSO alone (Ctrl), 10 μ M of progesterone (Prog), 10 μ M of lynestrenol (Lyn) or 1 μ M of GW3965 (GW), with or without 5 μ M RU486 for another 72h. Western blotting (A) was used to measure media apoE (B), cellular protein levels of apoE (C) and ABCA1 (D) in whole cell lysates. Band intensity was quantitated by densitometry and expressed as fold change relative to the DMSO control without RU486 co-treatment. Real-time quantitative PCR was used to measure apoE (E) and ABCA1 (F) mRNA levels in whole cell lysates. Data represent mean and SD of fold differences from six independent experiments for B, five experiments for C and D, three experiments for E and F. Drug effect of progestins and GW3965 was analyzed by two-way ANOVA with a Tukey's post test. Effect of RU486 was analyzed by two-way ANOVA with a Sidak's column comparison. * represents $p < 0.05$, ** represents $p < 0.01$, and *** represents $p < 0.0001$.

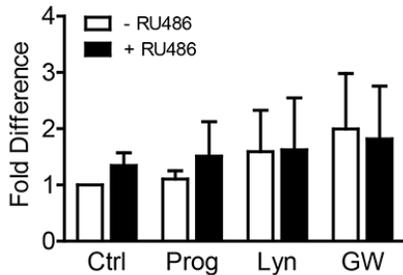
A ApoE3-expressing astrocytes



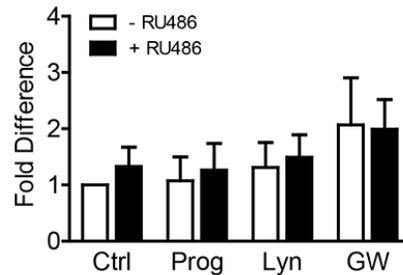
B ApoE4-expressing astrocytes



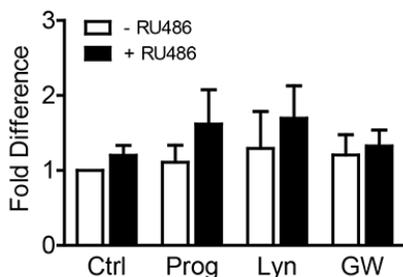
C E3: Media apoE



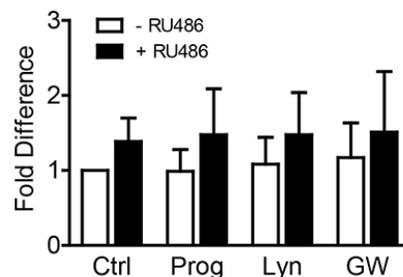
D E4: Media apoE



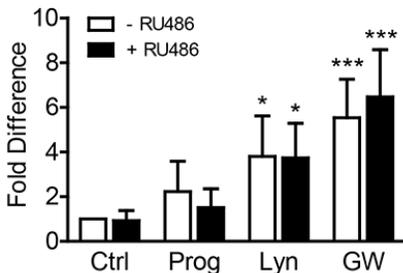
E E3: Cellular apoE



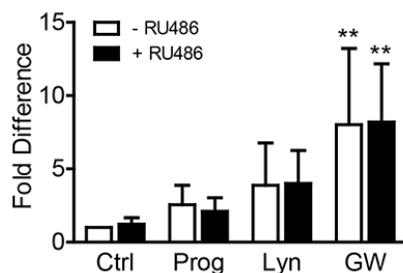
F E4: Cellular apoE



G E3: Cellular ABCA1



H E4: Cellular ABCA1



progesterone and GW3965 was analyzed by two-way ANOVA with a Tukey's post test. Effect of RU486 was analyzed by two-way ANOVA with a Sidak's column comparison. * represents $p < 0.05$, ** represents $p < 0.01$, and *** represents $p < 0.0001$.

Supplemental Figure A.3: Effects of progesterone and lynestrenol in apoE3- and apoE4-expressing immortalized astrocytes.

Immortalized astrocytes from human apoE3 and apoE4 knock-in mice were seeded in 12-well plates and pre-treated with 5 μM of RU486 or DMSO alone for 1h, followed by treatment of DMSO alone (Ctrl), 1 μM of progesterone (Prog), lynestrenol (Lyn) or GW3965 (GW) with or without 5 μM RU486 for another 24h. Western blotting (A, B) was used to measure media apoE (C, D), cellular protein levels of apoE (E, F) and ABCA1 (G, H) in whole cell lysates. Band intensity was quantitated by densitometry and expressed as fold change relative to the DMSO control without RU486 co-treatment. Data represent mean and SD of fold differences from three independent experiments for C-F, four experiments for G and H. Drug effect of

Appendix B Important contributions in co-authored papers

B.1 ABCA1 mediates the beneficial effects of the LXR agonist GW3965 on object recognition memory and amyloid burden in APP/PS1 mice.¹

In this study, Donkin et al. performed the first examination of the specific role of ABCA1 in mediating beneficial responses to LXR agonists in AD mice. The authors evaluated behavioral and neuropathological outcomes in LXR agonist, GW3965, treated female APP/PS1 mice that either express or lack ABCA1. GW3965 treatment increased both ABCA1 and apoE protein levels in the brain of APP/PS1 mice. Moreover, ABCA1 was required to observe significantly induced apoE levels by GW3965 in brain tissue and in cerebrospinal fluid (CSF). APP/PS1 mice that express ABCA1 showed a clear trend toward reduced amyloid burden in the brain when treated with either low dose (2.5/mg/kg/day) or high dose (33 mg/kg/day) of GW3965. Conversely, APP/PS1 mice lacking ABCA1 failed to display reduced amyloid load in the whole brain and showed trends toward increased hippocampal amyloid upon GW3965 treatment. Treatment with either dose of GW3965 also completely restored novel object recognition memory in the APP/PS1 mice to wild-type levels, which also requires ABCA1. My contribution to this work consisted in the extraction of murine CSF and evaluation of the CNS-derived apoE lipoprotein in CSF on native PAGE assay (**Fig. B.1**).

As described in Chapter 1, in amyloid mouse models of AD, ABCA1 deficiency exacerbates amyloidogenesis whereas ABCA1 overexpression reduce amyloid load, suggesting an important

¹ A version of this section has been published. Donkin JJ, Stukas S, Hirsch-Reinshagen V, Namjoshi D, Wilkinson A, May S, Chan J, Fan J, Collins J, Wellington CL. ATP-binding cassette transporter A1 mediates the beneficial effects of the liver X receptor agonist GW3965 on object recognition memory and amyloid burden in amyloid precursor protein/presenilin 1 mice. *J Biol Chem.* 2010 Oct 29;285(44):34144-54.

role of ABCA1 in A β metabolism. LXR agonists including GW3965 upregulate transcription of several genes including ABCA and apoE and have been shown to improve memory and reduce brain A β levels, particularly hippocampal A β 42 in AD mice (Burns et al., 2006b; Q. Jiang et al., 2008; R. P. Koldamova et al., 2005; Riddell et al., 2007; Vanmierlo et al., 2009). However, the specific role of ABCA1 in mediating beneficial response to LXR agonists in AD mice had not been evaluated before this study. Determining which LXR target genes mediate the beneficial effects of LXR agonists on behavior and neuropathology may offer insights into new strategies that avoid the undesirable side effects of current synthetic LXR agonists. Thus, the objective of this study is to assess the specific contribution of ABCA1 in the beneficial response to LXR agonists in APP/PS1 mice.

The authors generated APP/PS1 mice with and without functional ABCA1 and treated female cohorts with the LXR agonist GW3965 using low dose (2.5 mg/kg/day) prophylactic, low dose (2.5 mg/kg/day) therapeutic, or high dose (33 mg/kg/day) therapeutic treatment arms. Mice in the prophylactic group were treated with GW3965 from 16 weeks up to 40 weeks of age (24-week duration), whereas mice in the therapeutic groups were treated from 32-40 weeks of age (8 week duration). Behavioral analyses were performed at 40 weeks of age followed by tissue harvesting. Western blotting analyses on the cortical and hippocampal tissue revealed that GW3965 increased ABCA1 and apoE protein levels in a dose-dependent manner but did not affect APP levels in the ABCA1-expressing AD mice. As predicted from previous studies, reduced apoE protein levels were observed in the ABCA1-deficient animals. The authors observed a subtle but insignificant increase in apoE protein levels in GW3965-treated ABCA1-deficient animals, as apoE is independently activated by GW3965. However, none of the GW3965 treatment

strategies was sufficient to restore apoE levels in the ABCA1-deficient animal to the wild-type levels observed in untreated mice, suggesting that increased transcription of apoE mRNA maybe offset by catabolism of poorly lipidated apoE protein in ABCA1-deficient animal.

My contribution to this work was to evaluate CSF apoE levels in response to GW 3965 treatment. Murine CSF was collected as described in section 4.1.1. On average, approximately 15 μ l of CSF was obtained per mouse and CSF samples were then analyzed by 6% non-denaturing (native) PAGE followed by apoE immunoblotting. We observed that treatment of APP/PS1 mice with functional ABCA1 with low dose of GW3965 have slightly increased CSF apoE levels, consistent with modestly elevated apoE protein levels in brain tissue. AD animals receiving high dose of GW3965 showed a dramatic 10-fold increase in lipidated CSF apoE particles sized from 10 nm to 17 nm (**Fig. B.1**). As predicted, untreated ABCA1-deficient AD mice displayed greatly reduced CSF apoE levels relative to the AD but ABCA1-expressing mice. Furthermore, parallel to the observation in brain tissue analysis, ABCA1-deficient AD mice completely failed to exhibit elevated apoE levels in CSF under any treatment strategy.

The authors then showed that high dose GW3965 shifted A β from fibrillar pools to soluble fractions, an effect that was independent of ABCA1. We measured carbonate-soluble A β 40 and A β 42, which represent monomeric A β peptide pools, and guanidine-soluble A β 40 and A β 42, which represent fibrillar pools of A β . Low dose GW3965, regardless of prophylactic or therapeutic, had little effect on either carbonate-soluble or guanidine-soluble pools of A β in the cortex or hippocampus. These results agreed with a previous study reporting that less than 30mg/kg/day of the LXR agonist TO901317 for 7 days does not alter A β levels in Tg2576 AD

mice (Riddell et al. 2007). On the other hand, our study found that APP/PS1 mice treated with high dose GW3965 for 8 weeks showed elevated carbonate-soluble A β 40 and A β 42 in both cortex and hippocampus, whereas the corresponding cortical guanidine-soluble A β fractions tended to decrease. Similar trends were observed in ABCA1-deficient APP/PS1 mice treated with high dose GW3965. These observations suggest that treatment of APP/PS1 mice with a high dose of GW3965 might promote the retention of A β in soluble rather than deposited pools irrespective of ABCA1. Our study also showed that GW3975 treatment tended to reduce amyloid load, stained by thioflavin-S histology, in the hippocampus and whole brain in the treated APP/PS1 cohorts with functional ABCA1. Conversely, ABCA1-deficient APP/PS1 mice failed to exhibit lowered amyloid levels in whole brain even when treated with high dose GW3965. In contrast, a significant increase of hippocampal amyloid load was observed in ABCA1-deficient AD mice receiving high dose of GW3965. These observations suggest that ABCA1 is required to the reduced amyloid burden in response to GW3965 treatment.

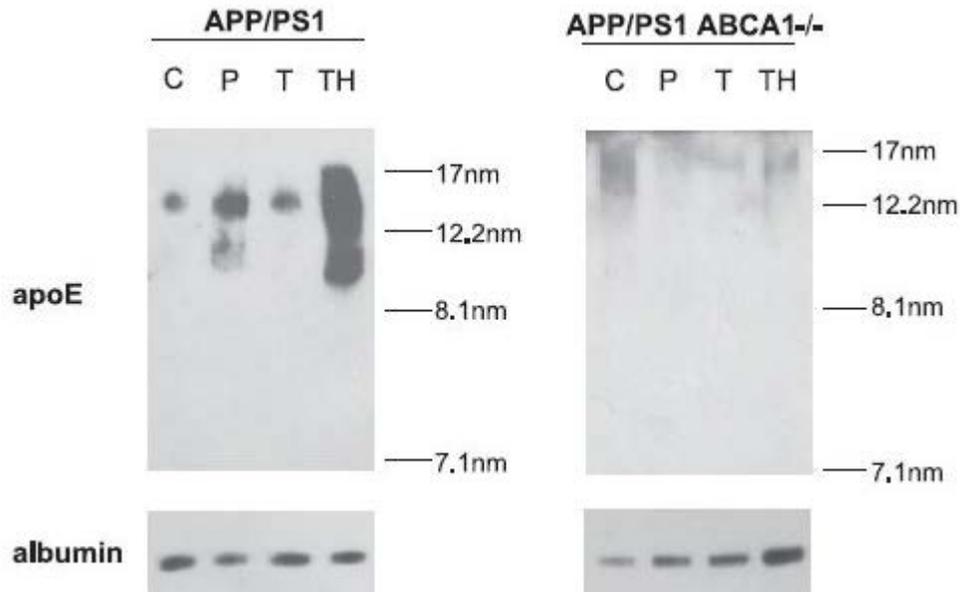


Figure B.1: ABCA1 is required for increased CSF apoE levels in response to GW 3965 in APP/PS1 mice.

Equal volumes of CSF from individual mice from untreated control (C), prophylactic (P), low dose therapeutic (T), and high dose therapeutic (TH) groups of APP/PS1 and APP/PS1 ABCA1^{-/-} mice are separated by 6% native PAGE and immunoblotted for apoE and albumin. Stokes diameter markers are shown in nm.

Novel object recognition (NOR) and Morris water maze (MWM) tasks were performed to assess the memory and cognitive performance of the AD animals in response to the LXR agonist treatment. In hippocampal-dependent NOR tests, all animals showed equivalent responses in training phase of the task, indicating that the presence of amyloid or the deficient of ABCA1 did not affect baseline exploratory behavior. As expected, wild-type control animals showed a significant preference for novelty, whereas untreated AD mice were considerably impaired in recognizing the novel object. Interestingly, all GW3965 treatment strategies significantly improved NOR performance of AD mice nearly to the wild-type levels, demonstrating that the hippocampal and perirhinal cortical pathways could respond positively to LXR agonist treatment

even after the onset of amyloid deposition. However, no treatment strategy was able to rescue the impaired NOR performance in the ABCA1-deficient APP/PS1 mice, clearly showing that ABCA1 deficiency compromises gains in cognitive function in response to GW3965 treatment. On the other hand, ABCA1 deficiency had subtler effects on MWM performance than for the NOR task. The impact of ABCA1 deficiency was only evident upon high dose therapeutic treatment, where lack of ABCA1 was associated with significantly poorer performance compared to the treated ABCA1-expressing AD mice, suggesting that neuronal pathways required for improvement in MWM task may be less responsive to LXR agonists compared to NOR task.

Together, the data presented in this manuscript show that ABCA1 is an important LXR target gene that contributes to positive effects of LXR agonists in AD mice. AD animals lacking ABCA1 completely failed to respond to GW3965 with regards to NOR performance and induction of tissue and CSF apoE levels. ABCA1 is also implicated in the tendency to reduce amyloid load in GW3965-treated AD mice. Our study also suggests that clinical improvement in cognitive functions may be possible without necessarily reducing total A β or amyloid levels. In summary, this manuscript is the first to specifically demonstrate ABCA1 as a key component of LXR beneficial response. Without ABCA1, apoE may not carry out its normal beneficial functions in CNS. Thus, therapeutic strategies that specifically target ABCA1 may be an alternative approach for bypassing the detrimental side effects of existing LXR agonist for AD.

B.1.1 Mouse cerebrospinal fluid collection

Prior to CSF collection, mice are anesthetized with a mixture of 20 mg/kg of xylazine and 150 mg/kg of ketamine via intraperitoneal injection. Once the animal is deeply anesthetized, a piece of skin between ears is removed to expose the neck musculature at the base of the skull. Three layers of musculature from the base of the skull to the first vertebrae are then carefully removed to expose the meninges overlying the cisterna magna. Bleeding vessels from the surrounding muscular tissues are cauterized to prevent blood contamination. The area above the cisterna magna is then gently cleaned with the use of cotton swabs to remove any residual blood. A 30G needle is used to puncture the arachnoid membrane covering the cisterna. The CSF will immediately begin to flow out the needle entry site under the positive pressure. A polypropylene narrow bore pipette is used to collect CSF as it exits the compartment. After the initial primary collection, the cistern will refill with several microliters more of CSF within two minutes. A second collection can then be performed to increase the net yield.

B.2 The LXR agonist GW3965 improves recovery from mild repetitive traumatic brain injury in mice partly through apolipoprotein E. ²

In this study, Namjoshi et al. evaluated the therapeutic potential of LXR agonist on traumatic brain injury (TBI) in the presence and absence of apoE. Male wild-type (WT) and apoE-deficient (apoE^{-/-}) mice received two consecutive mild repetitive traumatic brain injuries (mrTBI) spaced 24h apart followed by treatment with vehicle or the LXR agonist GW3965 at 15 mg/kg/day following the second injury. Behavioral, biochemical and histological outcomes were then measured. Data showed that GW3965 treatment restored the impaired NOR memory in WT but not in apoE^{-/-} animals, clearly indicating apoE is critical for the LXR agonist to exert its beneficial effect on NOR performance. However GW3965 did not enhance the spontaneous recovery of motor deficits in all groups, suggesting the LXR agonist does not affect recovery of motor impairment following mrTBI. Our model of mrTBI induced the levels of soluble murine A β 40 and A β 42 in both WT and apoE^{-/-} mice. Interestingly, GW3965 treatment suppressed the elevation of A β in both genotypes. WT animals exhibit mild axonal damage after 2 days of mrTBI which was suppressed by GW3965. In contrast, apoE^{-/-} mice exhibited severe axonal damage that is unresponsive to GW3965 treatment. Together, data from this study suggests that both apoE-dependent and apoE-independent pathways contribute to the ability of GW3965 to enhance recovery from mrTBI. My contribution to this work consisted in the western blot analysis evaluating ABCA1 protein level changes by LXR agonist treatment after mrTBI (**Fig.**

² A version of this section has been published. Namjoshi DR, Martin G, Donkin J, Wilkinson A, Stukas S, Fan J, Carr M, Tabarestani S, Wuerth K, Hancock RE, Wellington CL. The liver X receptor agonist GW3965 improves recovery from mild repetitive traumatic brain injury in mice partly through apolipoprotein E. PLoS One. 2013;8(1):e53529.

B.2), as well as the extraction of mice CSF and evaluation of CSF apoE and apoA-I levels using immunoblotting (**Fig. B.3**, data not published).

Traumatic brain injury is a leading type of injury that causes death and disability with an estimated annual incidence of 500 cases per 100,000 persons in North America (Corrigan et al., 2010). The physical forces during TBI deform brain tissues, which causes a primary injury that directly affect neurons, axons, glial and blood vessels in a focal, multifocal or diffused pattern. The primary injury then initiates a cascade of secondary processes that lead to complex cellular, inflammatory, neurochemical and metabolic alterations after TBI (Werner & Engelhard, 2007). Unlike the primary injury, to which few treatment options are currently available, secondary injury pathways are potentially treatable. ApoE is one of the potential therapeutic targets for TBI as it has been shown to play critical roles in the neurobiological changes after TBI. Brain injury increases apoE levels in order to scavenge lipids released by degenerating neurons and myelin and to re-deliver lipids later during neuronal repairing, reinnervation and synaptogenesis (Laskowitz et al., 1998; Poirier, 1994). In mice, apoE deficiency compromises recovery from acute neurological injuries (Y. Chen et al., 1997; Han & Chung, 2000). Several studies report that APOE ϵ 4 carriers have significantly poorer outcomes compared to non-carriers (Lichtman et al., 2000; Smith et al., 2006; Teasdale et al., 1997), though two other studies find no association (Y. Jiang et al., 2008; Pruthi et al., 2010). A meta-analysis of 14 studies with 2527 subjects concludes that apoE4 does not affect initial injury severity but may compromise recovery at 6 months post-TBI (Zhou et al., 2008). Interestingly, several epidemiological studies suggest that TBI may increase the risk of AD, although the association is not always observed. It is noteworthy that APP accumulation in damaged axons is a striking histological hallmark of

diffuse axonal injury (Gentleman et al., 1993; Sherriff et al., 1994). Increased APP in the post-TBI brain could trigger A β production that leads to the amyloid cascade similar in AD. Microdialysis experiments in brain-injured humans demonstrate that ISF A β levels correlate positively with patient's symptom score, suggesting that A β release is associated with recovery of neuronal function (Magnoni & Brody, 2010; Magnoni et al., 2012; Schwetye et al., 2010). Therefore, it is hypothesized that improving apoE function may facilitate both neuronal repair and A β clearance after TBI, offering potential acute and long-term benefits. LXR agonists that regulate the transcription of many genes involved in lipid metabolism and inflammation have been shown to have beneficial effects in treating AD mouse models, one example being the manuscript summarized in section 4.1. Thus, it is worthwhile to test the possible therapeutic potential of LXR agonists for TBI. Since approximately 80% of human TBI are mild injuries without skull fracture and loss of consciousness (Decuyper & Klimo, 2012), the main objective of this study is to evaluate the therapeutic utility of LXR agonist GW3965 in treating a mild repetitive TBI mouse model.

The authors established a mouse model of mTBI that used a gravity-driven weight drop device to deliver two consecutive head injuries 24h apart. Injured WT and apoE^{-/-} mice were randomized into treated and untreated groups (WT: N=45/group, apoE^{-/-}: N=30/group). The treated groups received an intraperitoneal (i.p) bolus of GW3965 (20mg/kg) 30 min after the 2nd impact. GW3965 treatment was then continued by feeding mice with standard rodent chow in which GW3965 was compounded at 120 mg/kg, which resulted in an average daily dose of 15 mg/kg, until the end of the study. Injured mice in the untreated group received a single i.p bolus of DMSO vehicle at equivalent volume 30 min after the 2nd injury. These mice were then fed

with standard rodent chow. Sham-operated (uninjured) mice received neither GW3965 nor vehicle.

The authors showed that apoE is required for GW3965 to improve cognitive function after mrTBI. NOR task was performed to assess the non-spatial working memory at 2, 7 and 14 days following mrTBI. All animal groups exhibited an equivalent time exploring the two identical objects, indicating that baseline exploratory behavior was not affected by genotype, surgical procedures or drug treatment. Compared to sham-operated animals, untreated injured WT and apoE^{-/-} mice failed to discriminate between the novel and familiar objects at all post-mrTBI time points. On the other hand, object recognition in GW3965-treated injured WT mice was restored by day 7 and 14 post-mrTBI. However, GW3965 treatment failed to improve NOR performance in apoE^{-/-} injured mice at any post-mrTBI time points, clearly demonstrating the importance of apoE in exerting the beneficial effect of GW3965 to improve cognition. Interestingly, GW3965 did not enhance the spontaneous recovery of motor deficits in all groups, suggesting that the beneficial effect of LXR agonists may be selective to neuronal circuits that are responsible for memory and learning. As predicted by previous studies, loss of apoE led to significantly more severe motor impairment compared to WT animals in both untreated and treated groups.

The authors also found that GW3965 prevented mrTBI-induced elevation of endogenous murine A β levels. However, the effect was apoE-independent. Total soluble A β 40 and A β 42 levels were significantly elevated in WT and apoE^{-/-} mice after injury, though the 1.5-fold increase in A β 40 was sustained during 14 days whereas the 1.7-fold increase in A β 42 level was transient and returned to baseline at day 14 post-injury. In contrast to untreated controls, GW3965 suppressed

the increase of both A β 40 and A β 42 in WT mice as compared to the sham. ApoE^{-/-} mice exhibited a similar sustained increase of A β 40 and a transient increase of A β 42. Intriguingly, GW3965 also effectively suppressed the increase in both A β 40 and A β 42 levels in apoE^{-/-} mice. Furthermore, loss of apoE did not lead to significantly greater accumulation of A β species after injury, nor did it affect the APP processing.

Histological assessment using silver staining revealed that WT mice exhibited mild but significant axonal damage in several white matter tracts including the corpus callosum, cingulum, external capsule and internal capsule at 2 d post-mrTBI but returned to baseline by 7 d, suggesting that neuronal repair pathways were efficiently activated in these regions. On the other hand, apoE^{-/-} mice showed more severe and sustained axonal damage in these regions from 2 to 14 d after mrTBI. GW3965 effectively suppressed axonal damage in all regions examined in WT mice. In contrast, the axonal damage in apoE^{-/-} mice was unresponsive to GW3965, again suggesting a critical role of apoE in axonal repair.

To determine whether lipid mobilization pathways were induced by this mrTBI model, the authors then measured the protein levels of ABCA1, apoE and LDLR in the mouse brains by immunoblotting. Interestingly, mrTBI itself induced a transient 1.6-fold increase of ABCA1 in WT animals at 7 d which returned to baseline by 14 d. As predicted, GW3965 augmented this response, resulting in a 2.4-fold increase in ABCA1 protein levels at 7 d in treated WT mice as compared to sham controls (**Fig. B.2**). These results are consistent with our previous findings of the ABCA1 induction in GW3965-treated APP/PS1 mice (section 4.1). In contrast to WT mice, untreated injured apoE^{-/-} mice did not exhibit a significant elevation of ABCA1 after mrTBI,

suggesting that the endogenous signaling for ABCA1 induction after injury may be compromised in the absence of apoE. Nevertheless, similar to the WT mice, ABCA1 protein levels were significantly increased by GW3965 treatment in the treated apoE^{-/-} mice, suggesting that GW3965 could directly regulate ABCA1 expression.

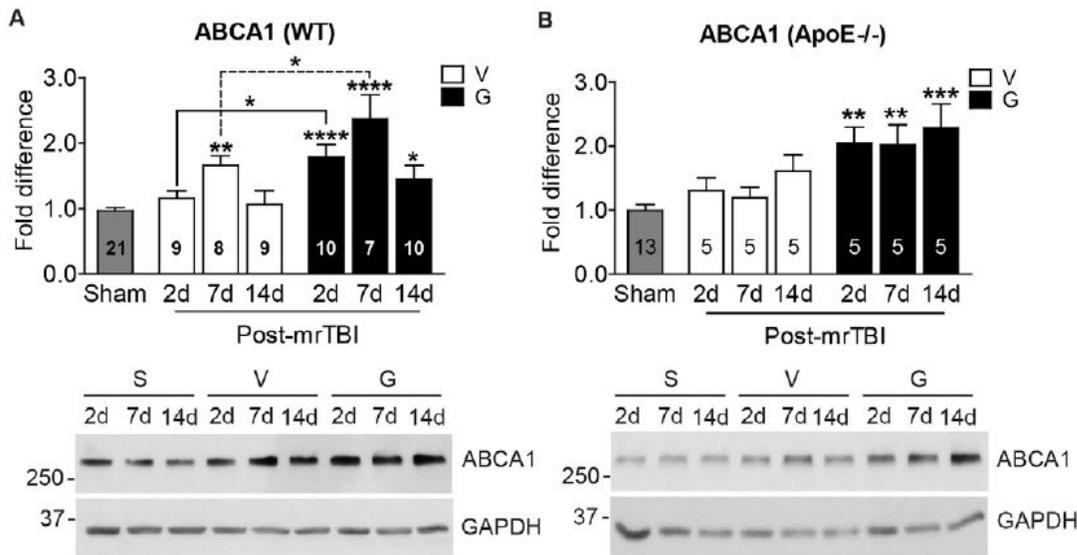


Figure B.2: GW3965 induces ABCA1 levels in WT and apoE^{-/-} mice following mild repetitive TBI.

ABCA1 protein levels were measured in ipsilateral half brains of WT (A) and apoE^{-/-} (B) mice post-mrTBI using Western blots. Data represents the fold difference normalized to sham control (grey bars). Numbers inside the bars indicate sample size. Data was analyzed by two-way ANOVA with a Bonferroni post hoc test. S: sham; V: untreated mic (open bars); G: GW3965-treated mice (black bars). *: p<0.05; **: p<0.01; ***: p<0.001.

Neither TBI nor GW3965 treatment resulted in significant changes in apoE levels in WT mice under our assay conditions, again suggesting that apoE is a relatively insensitive LXR target. Similar to apoE, the apoE receptor LDLR levels remained unchanged by mrTBI or GW3965 treatment. Intriguingly, even though CSF apoE was also unchanged in all groups, CSF apoA-I seemed to be effectively increased in GW3965-treated WT mice at 14 d as compared to sham

group and 2d treated group (**Fig. B.3**), implicating apoA-I being another LXR target in CNS. This piece of data, however, was not included in the published manuscript for its incompleteness due to the technical difficulty in CSF collection that prevented the authors from obtaining enough sample size, especially for the apoE^{-/-} groups.

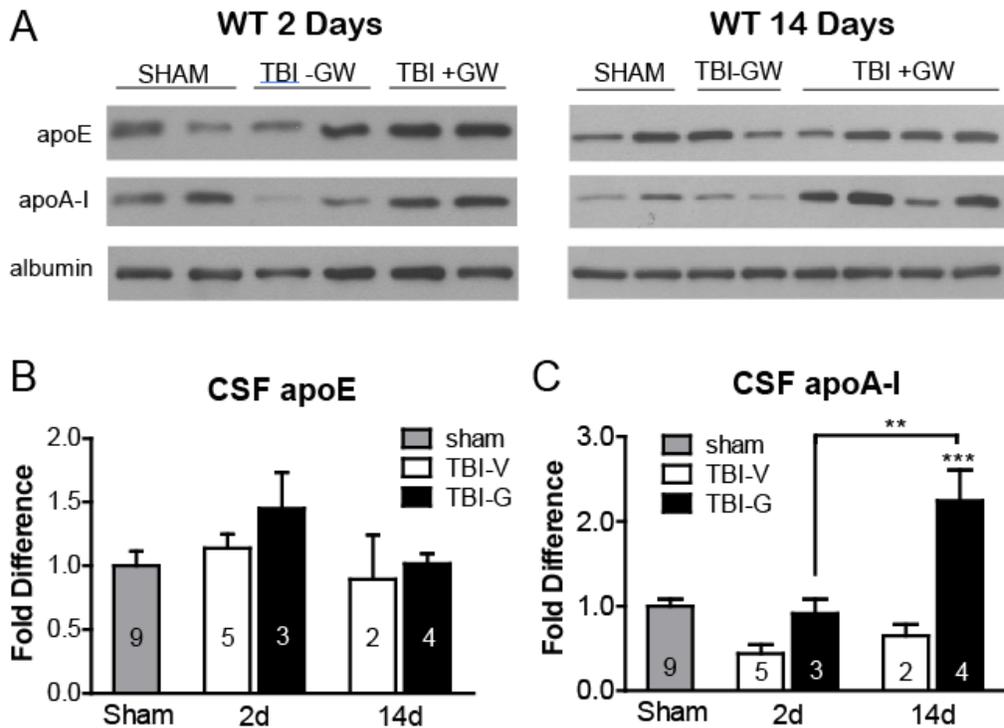


Figure B.3: GW3965 increases CSF apoA-I levels in WT mice following mrTBI.

8 μ l of mouse CSF was loaded on SDS-PAGE followed by apoE and apoA-I immunoblotting. Representative blots are shown in (A). Quantified data for CSF apoE (B) and CSF apoA-I (C) levels are expressed as fold difference normalized to sham values (grey bars). Numbers inside the bars indicate sample size. Data were analyzed by one-way ANOVA with a Bonferroni post hoc test. **: $p < 0.01$, ***: $p > 0.001$. V: untreated mice, open bars; G: GW3965-treated mice, black bars.

Together, the data presented in this manuscript show that the LXR agonist GW3965 could promote recovery in a mouse model of mild repetitive TBI that is designed to mimic repeated

concussion in humans. Therapeutic administration of GW3965 improved NOR performance, suppressed A β accumulation and reduced axonal damage after mrTBI. Both apoE-dependent and apoE-independent pathways contribute to the ability of GW3965 to promote recovery from mrTBI. Loss of apoE eliminated the ability of GW3965 to restore NOR performance and to promote axonal recovery, whereas apoE deficiency surprisingly did not hinder the ability of GW3965 to suppress elevated A β induced by injury. Although overall brain apoE levels remained unchanged after TBI, loss of apoE did exacerbate the severity of motor impairment and axonal damage, indicating the role of apoE in neuronal repair. It is possible that apoE may show localized upregulation in regions with more severe damage, which requires further investigation. ABCA1, the key transporter that lipidates apoE in brain, was found to be upregulated in WT mice after injury, suggesting that neuronal injury may promote the lipidation of apoE to scavenge lipids that are released during damage. Although apoE seems to be a less sensitive LXR target than ABCA1, we observe consistent upregulation of ABCA1 by LXR agonist GW3965 in mice. Future studies using ABCA1^{-/-} or ABCA1-overexpressing animal models are needed to test whether ABCA1-mediated lipidation of apoE contributes to the beneficial effects of GW3965 after mrTBI.

B.3 Specific loss of brain ABCA1 increases brain cholesterol uptake and influences neuronal structure and function.³

In this study, Karasinska et al investigated the specific role of ABCA1 in brain cholesterol homeostasis and trafficking by generating a mouse model in which CNS ABCA1 is specifically knocked out using the Cre/loxP recombination system. Unexpectedly, these mice exhibited reduced plasma HDL cholesterol levels despite wild-type levels of ABCA1 expression in peripheral tissues, which was associated with decreased brain cholesterol levels and enhanced CNS uptake of esterified cholesterol from circulating HDL. Increased levels of HDL receptor SR-BI was evident in brain capillaries. Moreover, CSF apoA-I levels was also elevated in the mutant mice. Phenotypically, disturbances in motor activity and sensorimotor function were observed, possibly due to the changes in synaptic ultrastructure, including reduced synapse and synaptic vesicle numbers, which were observed in the mutant mice. Together these data show that ABCA1 is a key regulator of brain cholesterol metabolism and that disturbances in cholesterol transport in the CNS are associated with structural and functional deficits in neurons as well as alterations in cholesterol uptake from plasma to brain. My contribution to this work consisted in the analysis of CSF lipoproteins by non-denaturing PAGE and immunoblotting (**Fig. B.4**).

Despite the important role of CNS cholesterol in synaptic function and neurodegenerative disorders, mechanisms regulating its transport and homeostasis are not well understood. The

³ A version of this section has been published. Karasinska JM, Rinninger F, Lütjohann D, Ruddle P, Franciosi S, Kruit JK, Singaraja RR, Hirsch-Reinshagen V, Fan J, Brunham LR, Bissada N, Ramakrishnan R, Wellington CL, Parks JS, Hayden MR. Specific loss of brain ABCA1 increases brain cholesterol uptake and influences neuronal structure and function. *J Neurosci*. 2009 Mar 18;29(11):3579-89.

cholesterol transporter ABCA1 has been shown to play critical roles not only in the peripheral HDL biogenesis but also in the CNS lipid homeostasis. ABCA1 knockout studies revealed that ABCA1 mediates cholesterol transport onto the CNS apoE and is critical for apoE secretion and lipidation (Hirsch-Reinshagen et al., 2004). However, in mice lacking ABCA1 globally, the specific role of brain ABCA1 cannot be fully elucidated without concurrent changes in whole body cholesterol homeostasis. Whole body ABCA^{-/-} mice have very low plasma HDL and apoA-I, depleting the possible pool of apoA-I that are available for transport into the CNS, which may have unknown effects on CNS cholesterol homeostasis. Indeed, specific deletion of ABCA1 in intestine and pancreas reveals tissue specific functions of ABCA1 that are not apparent in whole body ABCA^{-/-} mice (Brunham et al., 2007). Therefore, this manuscript was designed to elucidate the exclusive role of brain ABCA1 in CNS cholesterol homeostasis and behavioral function by developing a mouse model that lacks ABCA1 specifically in neurons and glia (ABCA1^{-B/-B}).

Karasinska et al first showed that while ABCA1 protein was absent in the brain, the levels of hepatic and intestinal ABCA1, which are critical contributors to plasma HDL levels, remained normal in the ABCA1^{-B/-B} mutant mice. However, they observed a significant reduction of cholesterol levels in plasma in addition to the reduced cholesterol levels in mutant cortex. Total cholesterol, HDL-cholesterol and phospholipid levels in plasma were all reduced in ABCA1^{-B/-B} animals, whereas the levels of triglycerides, which are associated with larger lipoprotein particles, remained unchanged. By using a radioisotope labeling technique that allows HDL metabolism to be monitored in vivo, the authors demonstrated that the significant decrease in plasma cholesterol levels were likely due to the increased active uptake of circulating HDL-

associated cholesterol esters into brain. Importantly, the integrity of the blood-brain barrier was not compromised in these ABCA1^{-B/-B} mutant animals. Since SR-BI receptor mediates the selective cellular uptake of cholesterol esters from HDL particles, the authors then measured SR-BI levels in isolated brain capillaries. Western blot analysis revealed a 2-fold increase in SR-BI protein levels in ABCA1^{-B/-B} mice, indicating that the receptor may indeed facilitate the increased brain cholesterol uptake in the mutant animals.

The authors then measured levels of brain apoE and apoA-I to determine the involvement of these apolipoproteins in the increased brain cholesterol uptake in ABCA1^{-B/-B} mice. Consistent with previous findings (Hirsch-Reinshagen et al., 2004), apoE protein levels in brain tissue were reduced in ABCA1^{-B/-B} mice. In contrast, the levels of brain apoA-I were significantly elevated (**Fig. B.4A**). Since apoA-I is generally not synthesized in the CNS (Elshourbagy et al., 1985) and no detectable levels of apoA-I mRNA in brains of ABCA1^{-B/-B} mice was found in this study, this increase is either due to increased apoA-I uptake from peripheral circulation or secretion from the brain capillary endothelial cells (BCECs). Analysis of CSF apoE- and apoA-I-containing lipoprotein particles by native PAGE revealed that CSF apoE levels were also reduced in ABCA1^{-B/-B} mice, whereas CSF apoA-I were considerably elevated. Interestingly, even though apoA-I levels are significantly increased in CSF of mutant animals, this apoA-I is predominately associated with small, less lipidated HDL particles in CSF (**Fig. B.4B**).

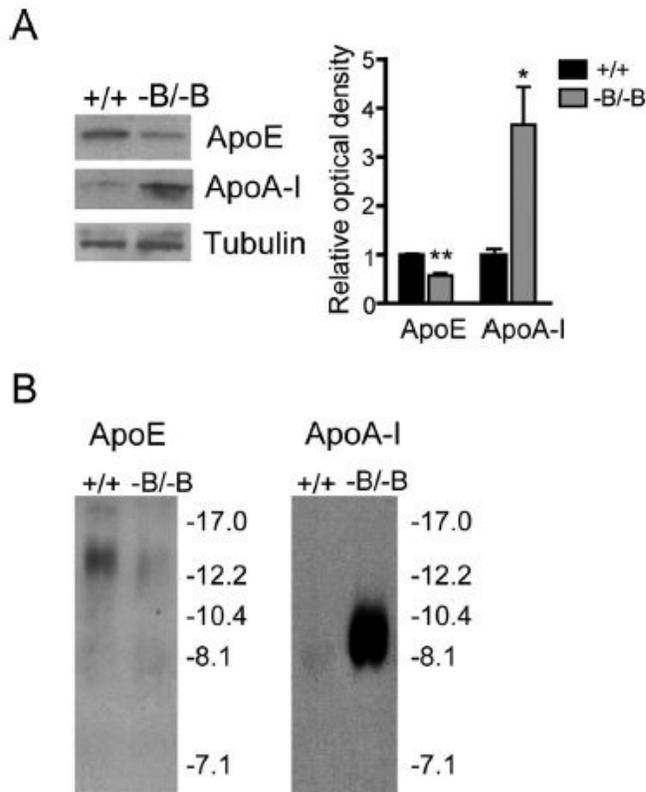


Figure B.4: ApoE and apoA-I levels in the brain and CSF of brain-specific ABCA1-knockout mice.

(A), Representative western blots and densitometry quantification of apoE and apoA-I levels in brain tissue from control and ABCA1^{-B/-B} mice. Tubulin was used as a loading control. Graph shows mean and SEM (*: p<0.05, **: p<0.01, N=3 per group). (B), 3 μ l of CSF samples were run on a native-PAGE and immunoblotted for apoE and apoA-I. Stokes diameter markers are shown in nm. Note: Panel (A) was generated by Joanna Karasinska.

The authors further demonstrated the abnormal behavior and synaptic structure found in ABCA1^{-B/-B} mice. Spontaneous locomotor activity was attenuated in 3-month old ABCA1^{-B/-B} mice in the open-field test. Stereotypic behavior and movement velocity were also reduced in mutant animals. Motor coordination by rotarod measurement was impaired in ABCA1^{-B/-B} animals at 9 month of age. Interestingly though, no changes in spatial learning in the Morris water maze were observed. ABCA1^{-B/-B} mice also exhibited a reduced response magnitude in acoustic startle test.

Together the behavior data indicate that specific loss of brain ABCA1 mainly affects motor and sensorimotor functions that are controlled by forebrain structures. Furthermore, immunofluorescence analysis of synaptotagmin, a marker for presynaptic vesicles, revealed a reduced intensity of synaptic puncta in cortex of mutant animals, indicating a possible presynaptic deficit. Electron microscopy examining the synaptic ultrastructure confirmed a decreased number of synapses, a reduced proportion of synapses containing a perforated postsynaptic density, and a decreased average number of synaptic vesicles per synapse in ABCA1^{-B/-B} mice, all suggesting that the attenuated locomotor activity in mutant mice could be associated with reduced number of excitatory synapses and vesicles in the cortex.

This study demonstrates that loss of brain ABCA1 activity can lead to not only changes in brain cholesterol homeostasis but also alterations in cholesterol transport across the blood-brain barrier. ABCA1^{-B/-B} mice exhibit increased brain uptake of esterified cholesterol from plasma HDL that is associated with reduced plasma HDL cholesterol levels. Loss of brain ABCA1 also leads to abnormal synaptic changes that may result in motor and sensorimotor deficits. Together, these results demonstrate a key role of ABCA1 in regulating brain cholesterol homeostasis and neuronal functions. This study also raises an important question as whether cholesterol transport across the blood-brain barrier is altered in neurodegenerative diseases. Reduced plasma HDL levels are reported in AD (Merched et al., 2000), whereas elevated 24S-OH cholesterol levels in plasma and CSF have also been observed in AD (Lutjohann et al., 2000; Papassotiropoulos et al., 2002; Schonknecht et al., 2002). Since brain ABCA1 function contributes to both CNS cholesterol homeostasis and cholesterol transport from peripheral, evaluation of brain ABCA1 as a therapeutic target in neurodegenerative disease should be considered in future studies.