EXPRESSION OF HUMAN ABCG1 IN MICE MODULATES
CHOLESTEROL METABOLITE LEVELS AND DOES NOT
INFLUENCE GUANIDINE-EXTRACTABLE ABETA LEVELS IN BRAIN

by

Braydon Burgess
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ABSTRACT

Growing evidence suggests that cholesterol plays a key role in Alzheimer's Disease (AD). Importantly, membrane cholesterol levels influence generation of the amyloid-beta (A\textbeta) peptide from amyloid precursor protein (APP). The amyloid cascade hypothesis of AD posits that increased accumulation of (A\textbeta) peptides and their deposition as amyloid in the brain, promote neurodegeneration in AD.

The ATP-binding-cassette-G1 (ABCG1) transporter is highly expressed in the brain and periphery and is believed to regulate cholesterol levels in peripheral cells and tissues. This suggests that the ABCG1-mediated effects on cholesterol homeostasis may influence A\textbeta production and AD. To determine the effect of ABCG1 overexpression in brain and to evaluate whether ABCG1 could influence A\textbeta production or amyloid deposition, ABCG1 overexpression was studied both in vitro and using a novel ABCG1 transgenic mouse.

We generated an ABCG1 overexpressing mouse containing the entire ABCG1 genomic locus and regulatory sequence, to promote "physiological" expression patterns of overexpression. We found that the ABCG1 transgene is physiologically regulated in vivo and overexpressed 3-6-fold at the protein level. Using a LacZ reporter driven by endogenous ABCG1 regulatory sequences, we showed that ABCG1 is highly expressed in hippocampal neurons. Importantly, ABCG1 overexpressing mice express the ABCG1 transgene in primary neurons and astrocytes.

Unexpectedly, the level of cholesterol biosynthetic precursors and 24S-hydroxysterol levels were reduced in adult ABCG1 overexpressing brain, suggesting a novel function of ABCG1. Total levels of cholesterol and other lipids were unaffected. In contrast to numerous published studies that studied constitutive overexpression of ABCG1 in vitro, overexpression of physiologically regulated ABCG1 in primary astrocytes did not facilitate cholesterol efflux to HDL. These experiments suggest that the role of ABCG1 in
vivo is not entirely understood and that ABCG1 may influence cholesterol biosynthesis or cholesterol precursor transport.

ABCG1 overexpression increased APP processing by β-secretase and the production of Aβ in transfected HEK cells. However, in vivo ABCG1 overexpression or deficiency did not significantly affect murine Aβ levels nor did overexpression affect amyloid formation or β-secretase mediated APP cleavage. These experiments suggest that ABCG1 overexpression does not impact amyloid formation as hypothesized.
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</tr>
<tr>
<td>ABCA1</td>
<td>ATP-Binding Cassette A1</td>
</tr>
<tr>
<td>AcLDL</td>
<td>Acetylated low-density lipoprotein</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's Disease</td>
</tr>
<tr>
<td>AICD</td>
<td>APP intracellular domain</td>
</tr>
<tr>
<td>Apo</td>
<td>Apolipoprotein</td>
</tr>
<tr>
<td>APP</td>
<td>β-Amyloid precursor protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>BACE-1</td>
<td>Beta-site APP cleaving enzyme-1</td>
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<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>beta-methylcyclodextrin</td>
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<td>C83</td>
<td>83-amino acid CTF</td>
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<tr>
<td>C99</td>
<td>99-amino acid CTF</td>
</tr>
<tr>
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<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CTF</td>
<td>C-terminal fragment of APP</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DS</td>
<td>Down Syndrome</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>FAD</td>
<td>Familial Alzheimer's Disease</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------------</td>
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<tr>
<td>GC:MS</td>
<td>Gas chromatography : Mass spectrometry</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>HMGR</td>
<td>3-hydroxy-3-methyl-glutaryl-CoA reductase</td>
</tr>
<tr>
<td>LAC-Z</td>
<td>Beta-galactosidase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver-X-receptor</td>
</tr>
<tr>
<td>LXRE</td>
<td>Liver-X-receptor response element</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary tangle</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-Steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF-β</td>
<td>Platelet derived growth factor-beta</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>Peroxisome proliferator-activated receptor -gamma</td>
</tr>
<tr>
<td>QRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid-X-receptor</td>
</tr>
<tr>
<td>sAPPα</td>
<td>Soluble APP from alpha cleavage</td>
</tr>
<tr>
<td>sAPPβ</td>
<td>Soluble APP from beta cleavage</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecylsulfate</td>
</tr>
<tr>
<td>Tg</td>
<td>Transgenic</td>
</tr>
</tbody>
</table>
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CO-AUTHORSHIP STATEMENT

The experiments presented in this thesis were planned and performed by the author with the following exceptions. QRT-PCR Measurements were performed by Jeniffer Chan. Total brain lipids were extracted by the author, however, quantification of lipid species were performed in collaboration with Dr. Sheila Innis, Department of Nutrition, University of British Columbia. Cholesterol metabolite levels were measured in collaboration with Dr. Dieter Lutjohann, Department of Clinical Pharmacology, University of Bonn. All studies involving HEK 293 cells were performed by the author, however, representative experiments shown were performed in conjunction with Gavin Tansley. Murine and human Aβ extraction and measurements were performed in collaboration with Dr. Ronald DeMattos, Lilly Research Laboratories, Indianapolis.
BACKGROUND

1.1. Alzheimer's Disease

1.1.1. Alzheimer's Disease – epidemiology and impact

Alzheimer's Disease (AD) is a neurodegenerative disease characterized by progressive loss of cognitive function and, eventually premature death. AD is the most common form of senile dementia affecting more than 4.5 million North Americans in 2003, triple the number diagnosed in 1980 and by 2050, more than 15 million people may be affected with AD. The average age of an individual diagnosed with probable AD is 73 years of age, and the average life span following a positive diagnosis is 4-6 years, or half of the average life expectancy. Current therapeutic options for mild to severe AD exist and fall into two classes: acetylcholinesterase inhibitors such as donepezil or galantamine or the NMDA receptor agonist memantine. Both classes of drugs target neurotransmitter dysfunction that occurs in AD, however, neither of these classes of drugs are considered “disease modifying” therapies. The benefits of these drugs are slight, resulting in short-term increases in measures of cognition, functioning and daily living that, in some studies, are ineffective at reducing patient care. The current drug regimens do not preclude the development of more effective treatments that by one estimate, could reduce the prevalence of AD by 50% if successful in delaying the onset of AD by 5 years.

1.1.2. AD and the brain

AD is associated with progressive impairments in memory, learning, judgment and changes in behavior. As the disease progresses, individuals lose the ability to care for themselves, and often become aggressive and exhibit signs of psychoses. In the final stages of AD, individuals are severely demented, often mute, and bedridden.
1.1.3. AD neuropathology

Behavioral tests are used to differentiate AD-related cognitive decline from other forms of dementia, and allow a diagnosis of possible or probable AD. A formal diagnosis of AD, however, is done post-mortem and requires histological evidence of the two hallmarks of AD neuropathology, which are extracellular amyloid plaques in the neural parenchyma and cerebrovasculature, and intracellular neurofibrillary tangles (NFT) of hyperphosphorylated tau protein. Cerebral atrophy from neuronal death is also observed at autopsy and by MRI in living patients with AD. The recent development of an amyloid-binding compound, Pittsburgh Compound-B (PIB), which is visible in positron emission tomography, has facilitated the detection of amyloid deposits in living patients. Comparison of PIB with cerebrospinal (CSF) fluid biomarkers suggest that quantifying amyloid in living patients may be effective at detecting AD prior to recognizable behavioral changes.

1.1.4. Tau

NFTs are intraneuronal deposits of filamentous protein aggregates, composed primarily of abnormally phosphorylated tau protein. Tau-protein is usually present in the axon, however, in some forms of dementia, including AD, tau protein becomes hyperphosphorylated forming cytoplasmic aggregates in the neuron. NFTs and hyperphosphorylated tau are observed in other forms of neurodegeneration such as Pick’s disease. Furthermore, mutations in tau are associated with an autosomal dominant form of frontotemporal dementia, suggesting that tau and NFTs are closely associated with and may be sufficient to cause dementia in the absence of amyloid. In AD, the density of NFTs in hippocampus and neocortex correlate well with dementia scores obtained shortly before death and an inverse correlation between NFTs and surviving neurites is reported. Taken together these studies suggest that NFTs play an important role in AD.
1.1.5. Amyloid plaques

Amyloid plaques are extracellular protein aggregates deposited around neurites and are associated with activated microglia. The core of the plaques are enriched in fibrillar peptides primarily of 40 or 42 amino acids in length and mature plaques contain a variety of aggregated proteins including Apolipoprotein E, Apolipoprotein J, anti-chymotrypsin and lipids. High concentrations of Aβ40 and Aβ42 (which possesses an isoleucine and alanine residue at its C-terminus) have been shown to spontaneously form amyloid fibrils in vitro. In vivo, histological analysis of newly formed plaques reveals that fibrillar Aβ42 is deposited first and may catalyze the aggregation of other proteins. Although amyloid fibrils have been shown to be directly toxic to neurons in vitro, more recent research suggests that the toxic Aβ species may be oligomeric Aβ, formed prior to deposition as amyloid. In addition to the direct toxicity of Aβ and amyloid, the propensity of amyloid to promote microglial activation may also be important in AD.

1.1.6. Amyloid and Inflammation

Histological investigation of the amyloid plaque in AD brains reveals that plaques are often infiltrated with activated microglia. In the brain, microglia mediate innate immunity in the CNS much like macrophages in the periphery. The consequence of microglial activation may be two-fold. First, activated microglia emit a respiratory burst designed to kill invasive microbes that releases neurotoxic free radicals into the parenchyma and can contribute to toxic oxidative stress upon the neurons. Secondly, microglia play an important role in the activation of complement, that promotes the formation of the membrane attack complex (MAC) on invasive cells leading to their lysis and death. Although the MAC is usually prevented from assembling on host cells, both amyloid and microglia secrete factors that have been shown to promote MAC formation and may lead to the accidental targeting of host cells. Histological analysis on dystrophic neurons in AD reveals that many of the apoptotic neurons are associated with MACs, suggesting that chronic microglia activation may be responsible for
much of the neuronal loss in the AD brain. The importance of inflammation in AD is underscored by retrospective studies that observed a 50% reduction in AD prevalence in arthritic individuals who chronically used NSAIDs. NSAIDs, which reduce the production of prostaglandin inflammatory mediators, are believed to be protective either by reducing the damaging effects of cerebral inflammation associated with AD, or by reducing the activity of γ-secretase.

1.1.7. The amyloid cascade hypothesis and familial AD

The amyloid cascade hypothesis of AD is the most widely accepted theory of AD, asserting that an increased abundance of Aβ in the brain and its eventual deposition as amyloid is the driving force behind other AD neuropathologies including NFT formation and neuron loss. The Aβ peptide was first sequenced in 1984 in a paper that reported that the primary component of amyloid in the AD brain were peptides deposited in a β-pleated sheet conformation. However, the strongest evidence supporting Aβ as a critical toxic mediator in AD came from genetic linkage studies of families afflicted with early-onset AD. In contrast to sporadic AD that accounts for ~95% of all reported AD cases, Familial AD (FAD) is associated with autosomal dominant mutations that cause AD between 35 and 60 years of age. When FAD mutations were first mapped to individual genes in the 1990s it was hypothesized that they would map to a gene containing the Aβ peptide sequence.

FAD mutations were found in amyloid precursor protein (APP), a large protein containing the Aβ peptide domain. However, these early studies found at least 3 FAD associated genes. Two well known FAD mutations in APP are the Swedish (KM670/671NL) and Indiana (V717F) mutations that both map to the boundaries of the Aβ peptide region. Other FAD mutations mapped to proteins with protease activity, presenilin-1 and presenilin-2 that were shown to cleave APP at the C-terminal boundary of the Aβ region. The convergent conclusion of these studies was that FAD mutations acted to increase production of Aβ from APP, or shift the production from the shorter 40 amino acid (Aβ40) peptide, to the
longer 42 reside form (Aβ42) \(^{37,38}\). Together these studies demonstrate that an increase in Aβ peptide production, specifically Aβ42, is sufficient to cause AD as early as 35 years of age, and formed the basis for the amyloid cascade hypothesis \(^{32,38}\). Although the cause of Aβ accumulation in FAD cases is well documented, the cause of Aβ accumulation in sporadic AD remains the subject of debate.

1.1.8. Amyloid Precursor Protein

The Aβ peptide is produced by proteolytic processing of the APP protein. APP is a type-I transmembrane glycoprotein existing in three main isoforms of 695, 751 and 770 amino acids \(^{39}\) and is expressed in many cell types in the body with very high expression in neurons. The APP protein shares sequence homology to well characterized proteins involved in cell signaling \(^{40}\) and in neurons, APP undergoes anterograde transport. APP is found in synapses, especially at sites of synaptogenesis \(^{41}\). It has been shown that APP forms signaling complexes with G protein coupled receptors involved in GTP-signaling pathways \(^{42}\) suggesting that APP may function as a membrane bound signaling receptor. Additionally, APP undergoes cleavage to liberate a soluble ectodomain (sAPP) that may act as a growth regulator in addition to a soluble intracellular domain (AICD) that co-localizes with the nucleus and is believed to be a transcriptional activator \(^{43,44}\). During the liberation of the two soluble domains from the extracellular and intracellular face of the membrane, the transmembrane spanning domain of APP remains associated with the membrane.

1.1.9. APP Processing

The processing of full-length APP into extracellular and intracellular domains depends upon the proteolytic activity of α, β and γ secretase (Figure 1-1) \(^{45}\). The cleavage of APP first occurs at the plasma membrane by either α or β secretase \(^{46}\). α-secretase cleaves APP within the Aβ region, to liberate the sAPPα domain and an 83 amino acid (C83) C-terminal fragment.
Proteolytic Processing of Amyloid Precursor Protein (APP)

Figure 1-1: Processing of the amyloid precursor protein by secretases. Full length APP undergoes cleavage at the plasma membrane by either (1a) α-secretase or (1b) β-secretase to produce a soluble extracellular domain and membrane bound CTF denoted C83 or C99. (2a) C83 is further cleaved by γ-secretase to produce non-pathogenic p3 fragments and the AICD. (2b) C99 is cleaved by γ-secretase to produce either Aβ40 or Aβ42 and the AICD. ## Location of the Swedish KM670/671NL mutation ** Indiana V717F mutation
(CTF) that traverses the plasma membrane. Because α-secretase catalyzes proteolysis within the Aβ region of APP, α-cleavage precludes the formation of Aβ. α-secretase activity is thought to localize to phospholipid-rich domains of the plasma membrane and may be achieved through the actions of multiple proteins shown to have α-secretase activity including TACE and ADAM10. Alternatively, the first step in APP processing can occur through cleavage by β-secretase-activity-enzyme 1 (BACE-1). The BACE-1 protein is an aspartyl-protease found in cholesterol-rich microdomains of the plasma membrane that cleaves APP to liberate the sAPPβ ectodomain and a 99 amino acid (C99) CTF. Mice deficient for BACE-1 do not generate Aβ, confirming that BACE-1 is essential to β-secretase cleavage. Following cleavage at the plasma membrane, C83 and C99 CTFs are internalized where they are degraded or further cleaved by γ-secretase to liberate the AICD. γ-secretase is a complex composed of presenilin-1, presenilin-2, nicastrin and APH-1 and cleaves C83 substrates within the phospholipid bilayer to release the soluble AICD from the transmembrane-spanning p3 peptide, or alternatively γ-secretase cleaves C99 to liberate the AICD and leave the Aβ peptide of either 40 or 42 amino acids associated with the membrane.

1.2. Cholesterol

1.2.1. Cholesterol in mammalian cells

Cholesterol is a 27 carbon molecule composed of 4 adjoining carbon rings, hydrocarbon tail and hydroxyl group. Cholesterol plays a key role in maintaining the proper fluidity of eukaryotic cell membranes and as a precursor for many hormones. The plasma membrane is enriched in cholesterol, possessing more than 80% of unesterified cholesterol in the cell and comprising 20-25% of the total lipid content of the plasma membrane. In health, cells balance their membrane cholesterol content by regulating its uptake, synthesis, storage and export, as excess membrane cholesterol can be cytotoxic. In the brain, neurons and glia import cholesterol and other lipids from lipoprotein-like particles containing apoE through receptors of the LDL-receptor family. Additionally, cells can synthesize their own cholesterol.
in the endoplasmic-reticulum (ER). The committed and rate limiting step in cholesterol biosynthesis is the enzymatic combination of 2 acetyl-CoA molecules to form mevalonate by 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR). Under conditions of low cellular cholesterol or mevalonate the transcription of HMGR is increased, whereas under conditions of high cholesterol the degradation of HMGR is increased. These properties allow the abundance of the HMGR protein to regulate cholesterol synthesis. Under conditions of excess cholesterol, plasma membrane cholesterol is either effluxed to extracellular acceptors or is returned to the ER where it can be esterified with fatty acids by AcetyCoA Transferase for storage in the hydrophobic core of cytoplasmic lipid droplets. Cells require cholesterol efflux to eliminate excess cholesterol as mammalian cells cannot degrade the sterol ring. To remove cholesterol from the body, cholesterol or sterol-derivatives such as oxysterols are returned to the liver for enzymatic conversion into bile-salts and bile-acids, which are ultimately eliminated in bile.

1.2.2. Cholesterol and the brain: The blood brain barrier

The brain accounts for approximately 2% of total body mass yet it contains 23% of the sterols in the entire body. It is estimated that 70-80% of the cholesterol in the brain surrounds neurons in myelin sheaths that maintain conductivity of the neuron, with the remainder present in cell membranes or lipoprotein-like particles in the CSF. In the periphery, cells obtain cholesterol from endogenous synthesis, or from circulating lipoproteins enriched in dietary cholesterol. However, the brain does not appear to import cholesterol from the peripheral circulation. This was best shown by an experiment that monitored uptake of isotopically labeled cholesterol given intravenously to baboons over 85 days. After 70-85 days all organs contained large quantities of labeled cholesterol, with the exception of the brain that acquired little or no cholesterol. Furthermore, experiments in mice fed a high-cholesterol diet found that while serum lipids were increased 7-8-fold above chow-fed mice, no evidence of cellular accumulation of cholesterol, or reduced synthesis was observed in the brain, in contrast
to peripheral organs \(^57\). The insulation of peripheral and brain cholesterol occurs at the Blood brain barrier (BBB), which consists of two layers of cells that surround capillaries of the brain. The first layer is a specialized subset of endothelial cells that form tight junctions with neighboring cells that prevent bulk-flow around the cells and show minimal rates of endocytosis and vesicular transport across their membranes \(^58\). The second line of defense is a sheath of astrocytic cell processes that surround the endothelial cells and, although they are essential for the impermeability of the membrane, their function is less well understood. This BBB, although impermeable to the influx of cholesterol, contains transporters to permit the flow of certain compounds such as glucose and is permeable to some hydrophobic molecules, including hydroxysterols that diffuse down concentration gradients across the BBB \(^50\). Because the brain is impermeable to cholesterol influx, all cholesterol must be endogenously synthesized.

### 1.2.3. Cholesterol and the brain: metabolism

In the adult brain many, if not all cells, have the capability to synthesize cholesterol, and to do so continuously (Figure 1-2) \(^50\). However, terminally differentiated adult neurons often do not synthesize sufficient cholesterol for processes such as axonal growth and synaptogenesis, and rely largely upon cholesterol obtained from glia. Because cholesterol cannot readily traverse the BBB into the periphery, cholesterol is eliminated from the brain mostly via conversion to the oxysterol 24S-hydroxycholesterol. 24S-hydroxycholesterol is not restricted by the BBB and passively diffuses down a concentration gradient into the circulation where it can be transported to the liver for elimination as bile acids \(^59\). 24S-hydroxycholesterol is produced enzymatically by Cyp46A1, which, in humans, is expressed exclusively in sub-sets of neurons \(^60\). The mechanism by which glial cells clear cholesterol is less well understood and may be coupled to cholesterol efflux to glial-derived apolipoproteins, as glia do not express Cyp46A1 \(^50,60\). Genetic polymorphisms in Cyp46A1 are associated with increased risk of AD in some studies \(^61\). However a meta-analysis of Cyp46A1 polymorphisms finds that only 11 of the 22 studies found a significant association between Cyp46A1 and AD and concluded that Cyp46A1 was not a significant contributor to AD in the general populace \(^62\).
Summary of the Cholesterol Biosynthetic Pathway

**HMGR**

\[ 2 \times \text{AcetylCoA} \rightarrow \text{Mevalonate} \]

\[ \text{Mevalonate} \rightarrow \text{Lanosterol} \]

\[ \text{Desmosterol} \rightarrow \text{Lathosterol} \]

\[ \text{DHCR24} \rightarrow \text{Cholesterol} \rightarrow \text{Cyp46A1} \rightarrow \text{24S-hydroxycholesterol} \]

**Figure 1-2:** Summary of the cholesterol biosynthetic pathway. Two molecules of AcetylCoA are converted to mevalonate by HMGR, in the first and rate limiting step of cholesterol biosynthesis. The pathway branches at lanosterol, through pathways involving desmosterol or lathosterol that are both precursors to cholesterol. In the brain, cholesterol is converted by Cyp46A1 to 24S-hydroxycholesterol that diffuses across the BBB for elimination into bile. Dotted lines represent multistep reactions, solid lines represent single step reactions and catalytic enzyme.
Extracellular cholesterol transport in the CNS, involves lipoprotein-particles of similar size and density to HDL, which are enriched in phospholipids and cholesterol and complexed with apoE and to a lesser degree apoJ \(^{63}\). CNS-lipoproteins are thought to be primarily formed by glia that synthesize the majority of the apolipoprotein in the brain \(^{63}\). In culture, the cholesterol delivered by apoE containing lipoproteins have been shown to dramatically increase the elongation of axons required in times of growth or neuronal repair and are required for efficient formation and maintenance of synapses \(^{64,65}\). These experiments suggest a central role of glia in brain lipid homeostasis, which is central to the growth, maintenance, and repair of neuronal membranes.

1.2.4. Cholesterol and Alzheimer’s Disease

Several converging lines of evidence suggest that the transport and levels of cholesterol in the brain may play an important role in the pathology of AD. Specifically, apoE genotype, serum cholesterol levels, and the therapeutic use of cholesterol biosynthesis inhibitors known collectively as statins, are implicated in the pathogenesis of AD.

1.2.4.1. Apolipoprotein E

The strongest genetic risk-factor for sporadic AD is allelic variations in the apolipoprotein E (apoE) gene, the main cholesterol carrying apolipoprotein in the brain. The human apoE locus is present in three main alleles, of which apoE3 is present in 70-78% of the population and is the most common allele, apoE4 is present in 20-30% of the population and apoE2 is the rarest allele, being present in only 7-8% of individuals \(^{66-68}\). It is well established that the apoE4 allele acts to decrease the average age of onset of AD relative to the apoE3 allele, and that a single allele of apoE2 exerts a protective effect. For example, apoE3/E2 carriers develop AD latest at an average of 84 years compared to an average of 68 years age in apoE4/E4 carriers. Furthermore, the prevalence of AD in non-apoE4 carriers at 75 years of age is 20% compared to 60% in the population carrying one-apoE4 allele, or 80% in apoE4/E4
carriers. These studies support the hypothesis that apoE4 promotes AD in a dose dependant manner.

The mechanisms by which substitutions in one or two amino acids among apoE2 (Cys112, Cys158), apoE3 (Cys112, Arg158) and apoE4(Arg112, Arg158) has been the focus of much research and has not reached consensus. It has been shown that apoE4 delivers less cholesterol to cultured neurons than apoE3, with apoE2 delivering the most. Neurite growth, particularly in the adult brain, is heavily dependant on apoE-derived cholesterol, and apoE4 may be less efficient at repairing damaged neurons in AD. An alternative line of evidence suggests that Aβ:apoE interactions may account for the impact of apoE genotype. ApoE3 but not apoE4 has been shown to form SDS-resistant complexes with Aβ that may impair the clearance of Aβ42 from the brain of apoE4 carriers via apoE receptors. Furthermore, apoE deficient mice do not form fibrillar amyloid, suggesting that apoE may catalyze the conversion of soluble Aβ into amyloid fibrils in vivo in an isoform specific manner. Although the mechanism underlying the apoE and AD interaction remains the subject of considerable debate, apoE remains the only well established genetic risk-factor for sporadic AD in the general population. Because sporadic AD accounts for more than 95% of AD cases, understanding how apoE contributes to AD pathogenesis may be of higher impact to worldwide disease burden than any treatments based on the pathways illuminated by FAD mutations that affect less than 5% of AD patients.

1.2.4.2. Serum Cholesterol

The second line of evidence supporting a role for cholesterol in AD are population studies that suggest elevated serum cholesterol is a risk factor for AD. The strongest evidence for this connection comes from studies with long follow-up periods. One study of 1449 individuals that obtained 4 measures of serum cholesterol from individuals over an average of 21 years found that cholesterol levels greater than 6.5mM in midlife were associated with 2.6 greater chance of being diagnosed with AD. This study confirmed that apoE isoform was a
genetic risk factor, but that the effect of serum cholesterol was independent of apoE. Similarly, the Finnish Male cohort study of the seven countries and the Honolulu aging study found that total cholesterol serum levels >6.5mM in midlife were associated with 3.1 greater risk of AD during a 15-25 year follow up period \(^76\)\(^-\)\(^78\). However, the Framingham study and Gotborg study, both with 10+ years of follow up concluded that there was either no association between total serum cholesterol and AD, or that higher cholesterol levels were associated with decreased dementia risk \(^79\),\(^80\). One criticism with the Framingham study however, with respect to the connection to AD, was the use of broadly stratified age groups that may not discriminate midlife measurements from older individuals. Numerous other studies have been performed, many with only 2-3 year follow up periods and, taken together, the number of studies reporting negative associations between markers of serum lipids and AD outnumber the positive association studies, reviewed \(^81\). Despite the contradictory results, the evidence suggests that cholesterol levels in midlife may be a better indicator of AD risk than cholesterol levels immediately prior to or upon diagnosis.

Studies using animal models have attempted to recapitulate the human studies with the use of high-cholesterol diets. Rabbits fed a diet high in cholesterol develop intraneuronal Aβ deposits in as few as 4 weeks of feeding, compared to chow-fed rabbits that have undetectable levels of Aβ \(^82\). Furthermore, when fed a diet high in cholesterol for 7 weeks the PSAPP mouse model of AD, which is transgenic for human APP and human mutant presnilin-1, was found to have larger and more numerous amyloid deposits than chow fed mice. The authors also found a positive correlation between total plasma cholesterol and insoluble Aβ load suggesting that a high dietary intake of cholesterol could influence APP processing and exacerbate amyloid deposition \(^83\). Although not all animal studies agree that hypercholesterolemia increases amyloid deposition, all studies have found evidence of altered APP processing in mice fed a high fat/cholesterol diet \(^84\),\(^85\). Taken together, these studies show that a high intake of dietary cholesterol can influence APP processing in the brain, supporting the hypothesis that high serum cholesterol may promote AD pathology in humans. The underlying mechanism behind
the correlation of high-serum cholesterol and AD is unclear however, as cholesterol in the central nervous system is believed to be insulated from peripheral sources.\textsuperscript{50}

1.2.4.3. Statins

The third line of evidence connecting cholesterol metabolism and AD is derived from retrospective studies finding that statin use was associated with a reduced prevalence of AD. Statins inhibit the activity of HMGR, which catalyzes the rate limiting step in cholesterol biosynthesis, and are prescribed to reduce LDL-cholesterol in patients at risk for cardiovascular disease. In the initial retrospective studies, the use of lovastatin or pravastatin was shown to reduce the prevalence of probable AD by 60-73\% when compared to either no-treatment or patients taking other medications for hypertension or cardiovascular disease.\textsuperscript{86} Despite the marked success of these early retrospective trials, the results of prospective trials have not reached a clear consensus. Two trials designed to validate the efficacy of simvastatin and pravastatin in hypercholesterolemic individuals 80 years of age or older, did not detect a difference in the incidence of AD between statin treated and non-treated individuals over a 5 year follow-up period.\textsuperscript{87,88} However, both of these prospective studies have received criticism for their use of phone interviews to assess AD during the follow up period, and may also have lacked the sensitivity to detect small changes in AD incidence rates.\textsuperscript{62} In contrast, a 2006 prospective trial conducted on individuals with early to mid stages of AD did find that atorvastatin improved cognition scores compared to placebo after 6 months and by a greater degree after 1 year.\textsuperscript{89} A slight beneficial effect of simvastatin on cognition was also confirmed in another prospective 26 week study of individuals with mild-moderate AD in the absence of preexisting hypercholesterolemia.\textsuperscript{90}

The discrepancies in statin studies may be related to the outcome measures used by individual studies. The prospective studies examining statin use in non-demented elderly failed to observe a significant impact on AD incidence, however, statin therapy was shown to improve cognitive markers in patients with mild AD. These studies suggest that while the initial benefit
statin therapy observed in retrospective trials may have been overestimated, statins may prove to be effective in treatment if not prevention of mild AD.

The mechanism by which statins may impact AD pathology is also the subject of debate. One line of evidence suggests that statins may reduce the production of Aβ in the brain. It was shown that hippocampal rat neurons exposed to statins secreted 60% fewer Aβ40 peptides in culture. In the same study, directly depleting membrane cholesterol using β-methylcyclodextrin (βMCD) produced a similar reduction in Aβ. Fassbender and colleagues went on to confirm their results in vivo, by demonstrating that Aβ40 and 42 levels in the brain of statin-fed guinea pigs was reduced by 40-60%. Similarly, HEK 293 cells treated with lovastatin, or βMCD, produced fewer Aβ peptides and had increased α-secretase activity. Both these studies, however, used very high statin doses, ~220-400x greater in guinea pigs than the average weight adjusted human dose. These experiments are interpreted to suggest that neuronal cholesterol levels modify APP processing, possibly by trafficking APP into BACE-1 containing cholesterol-rich lipid raft domains under conditions of abundant cholesterol, or by trafficking APP into phospholipid rich α-secretase containing membrane domains under conditions of low cholesterol. Accordingly, statins influence neuronal cholesterol levels and thus influence APP processing and Aβ production.

Despite the caveats of the animal studies, human trials suggest that statin therapy of therapeutic doses may have a similarly beneficial effect. Patients treated with simvastatin were shown to have reduced sAPPα and sAPPβ in CSF, suggesting that statin therapy reduces the processing of APP. Furthermore, individuals treated for 3 months with atorvastatin of various therapeutically approved doses were found to have a dose dependant decrease in plasma Aβ. Although plasma Aβ is not a reliable biomarker for AD and the significance of these results to AD is unclear, the studies provide evidence that statin treatment may reduce the production of Aβ in humans.

The role of statins in AD however, may be more complex than cholesterol-mediated effects on Aβ production. The HMGR enzyme produces the precursors for both cholesterol
and a class of molecules known as isoprenoids, and statin therapy inhibits the synthesis of both cholesterol and isoprenoids. Isoprenoids are involved in signaling, especially in mediating inflammatory reactions. Studies on rat brain-slice cultures show that statins strongly reduce microglial activation, and are especially effective at reducing Aβ-induced microglia activation. The anti-inflammatory effects of statins are also believed to be important for their protective role in atherosclerosis, suggesting that any beneficial effects on AD may be pleiotropic, resulting from both reduced synthesis of cholesterol and inhibition of isoprenoid-mediated inflammatory responses.

1.3. Down Syndrome and AD

The APP gene is located on chromosome 21 and triplication of chromosome 21 in Down Syndrome (DS) inevitably results in the rapid accumulation of AD-like neuropathology by the age of 40 years. Interestingly, the triplication of APP alone through small chromosomal rearrangements is also associated with early-onset AD. A recent phylogenetic analysis of five separate families with duplicated APP found that inheritance of three copies of APP was sufficient to cause AD-like dementia between 42 to 59 years of age in 21 out of 21 individuals studied, in the absence of DS. The presence of AD neuropathology was confirmed at autopsy. In comparison, severe AD-like neuropathology is observed in almost all DS cases at autopsy by 40 years of age, reviewed in. The specific age of onset for AD dementia in DS is difficult to assess because of the underlying and highly variable degree of mental retardation in DS. Because of this, the onset of AD-related dementia in DS cannot be directly compared to APP triplication alone. However, a comparison of the reported onset of pathology between the two studies suggests that AD neuropathology develops more quickly in the DS population. Almost 100% of DS individuals have pathology by 40 years of age versus APP triplication who develop dementia at an average of 52 years and as late as 59. This comparison suggests that other genes on chromosome 21 may play a role in AD when overexpressed in DS subjects.
1.4. ABC Transporters

1.4.1. The ATP-Binding-Cassette superfamily

The ATP-Binding-Cassette (ABC) superfamily of transporters contains 48 genes subdivided into 8 sub-classes classified from ABCA to ABCG. Typical ABC transporters consist of two sets of 6 transmembrane α-helix domains, dual ATP binding-domains and a characteristic signature motif specific to ABC transporters, reviewed in \(^{102}\). Eukaryotic ABC transporters are either full-size, containing all 12 α-helices on one polypeptide chain, or half-size requiring homodimerization, or heterodimerization with a similar ABC transporter to form a functional unit \(^{103}\). The prototypical function of an ABC transporter is to promote the uni-directional movement of compounds or peptides across lipid membranes using energy provided by the hydrolysis of ATP. ABC transporters typically transport their substrate from cytoplasm to extracellular space, or in the case of lipid transporters, from the inner leaflet of the plasma membrane to the outer leaflet or to an extracellular lipid acceptor \(^{102}\). Specific examples of ABC transporters include ABCB1 involved in multidrug resistance \(^{104}\), the nucleoside transporter ABCC4, and ABCA1 that transports cholesterol and phospholipids to Apolipopoprotein A1 to form high density lipoprotein (HDL) \(^{105}\).

1.4.2. The ABCG1 transporter

ABCG1 is a half-size transporter with a proposed role in sterol and lipid transport. Human \textit{Abcg1} is encoded on chromosome 21q.22.3, contains 23 exons and is 97% identical to murine ABCG1 at the protein level \(^{106}\). In humans \textit{Abcg1} is expressed in most tissues, and is especially abundant in liver, spleen, thymus and brain \(^{107}\). Primary sequence topology prediction reveals a typical ABC transporter design, with alternative splicing occurring in the N-terminal intracellular loop, and the constant exons (exons 11-23) encoding the canonical 6
ABCG1: Predicted Topology

Figure 1-3: Predicted topology of the ABCG1 monomer
Each ABCG1 monomer is predicted to encode 6 transmembrane alpha-helices and the Walker A and Walker B ATP binding domains that comprise the constant domain. The N-terminus of the protein is subject to alternative splicing and more than 11 isoforms have been detected in macrophages.

transmembrane helices and the Walker A and Walker B ATP binding domains (Figure 1-3)\textsuperscript{108}. As many as 11 alternatively spliced isoforms have been reported in macrophages, although only 4 of these have been validated\textsuperscript{106,109}. All identified transcripts possess one or more of the first 10 exons, and all of exons 11-23. The transcript corresponding to exons 5, 7 and 11-23 codes for a protein of 678 amino acids in length and shows the greatest induction to oxysterol treatment both in vitro and vivo\textsuperscript{106}. Overexpression of 4 different Abcg1 isoforms has been shown to have similar effects on sterol transport, however, the significance of the different isoforms, or the tissue specific expression patterns of different isoforms have not been well characterized.
1.4.3. Transcriptional regulation of Abcg1

Several ABC transporters involved in the regulation of sterol homeostasis are transcriptionally regulated by agonists of the Liver-X-Receptor (LXR) nuclear receptors \(^{110}\). The promoter region of the Abcg1 gene in both mice and humans possesses multiple LXR-Response-elements (LXREs) upstream of exon one and upstream of exon 8 \(^{106,109}\). LXRE-mediated oxysterol-sensitive transcription is achieved through the binding of LXR and RXR transcription factor dimers, which bind oxysterol and retinoid ligands, respectively, in the cytosol, and translocate to the nucleus where they bind LXREs and promote transcription \(^{110}\). Because oxysterol ligands are produced as cholesterol byproducts in cholesterol laden cells, LXRE responsive genes such as Abcg1 will be expressed under conditions of excess cholesterol suggestive of a role in cholesterol metabolism \(^{110}\). Functional LXRE sensitivity of the Abcg1 gene was confirmed by mRNA overexpression following treatment with 20S-hydroxycholesterol, 22R-hydroxycholesterol and the synthetic LXR-ligand TO901317, and ablation of expression in cells deficient for LXR transcription factors \(^{111}\). The Abcg1 gene is also regulated by the peroxisome proliferator-activated-receptor-gamma (PPAR-\(\gamma\)) transcription factor in vivo \(^{112}\). Abcg1 expression was induced in macrophages and aorta following treatment with the PPAR-\(\gamma\) ligand rosiglitizone, and this activation was shown to be independent of LXR transcription factors \(^{112}\). PPAR-\(\gamma\) activation suppresses the inflammatory response and is reported in activated macrophages and microglia \(^{113}\).

1.4.4. Transporter assembly

Because the ABCG transporters are half-transporters and ABCG5 and ABCG8, require heterodimerization to function, the assembly of functional ABCG1 transporters is an important consideration for overexpression studies. Human Abcg1 is considered a functional homologue of the Drosophila melanogaster white gene, which transports amino acids for pigment synthesis in the retina. Studies in D.melanogaster suggest that white may require heterodimerization with scarlet, another ABC protein, to form a functional transporter \(^{114}\). Accordingly, mammalian
ABCG1 was suspected to form heterodimers with a highly homologous transporter known as ABCG4, which was supported by one report suggesting that co-expression of ABCG1 and ABCG4 was required to promote ATP hydrolysis using an insect cell system\textsuperscript{115}. However, selective overexpression of ABCG1 alone in various mammalian cell types produced functional transporter activity, which was not augmented by co-expression with ABCG4\textsuperscript{116} suggesting that ABCG1 can form functional homodimers.

1.4.5. ABCG1 function

The established function of ABCG1 is to facilitate the efflux of cellular cholesterol to lipidated apolipoproteins such as HDL. Although the specific mechanism by which ABCG1 facilitates the transport of lipids remains elusive, the role of ABCG1 in lipid efflux has been studied in more than 30 peer reviewed articles between 2005 and 2007. The majority of these reports focus on sterol transport in the periphery, because of the importance of sterol-efflux to atherosclerosis, in which the export of cholesterol from lipid laden macrophages is thought to be the rate limiting step in preventing lesions in the vessel wall. In brief, the overexpression of Abcg1 cDNAs has been shown in various cell types to promote cholesterol efflux to HDL, but not to lipid-poor apolipoproteins, and that this activity is likely mediated by enriching the outer leaflet of the plasma membrane with free-cholesterol\textsuperscript{106,116,117}. ABCG1 deficiency in cells impairs efflux of cholesterol and choline-based phospholipids to HDL and, furthermore, ABCG1 deficient mice have been reported to accumulate more cholesterol and neutral lipids in a variety of tissues when fed a high fat diet compared to wildtype animals\textsuperscript{107,118}. In summary, ABCG1 in vivo appears to play an important role in maintaining tissue lipid homeostasis, likely though facilitating lipid export.

In contrast to ABCA1 that specifically transports lipids to poorly lipidated or lipid-free apolipoproteins, ABCG1 facilitates efflux to various lipid containing particles including discoidal apoAI, LDL, HDL and synthetic phospholipids vesicles, suggesting that the function of ABCG1 is distinct from ABCA1\textsuperscript{119,120}. 
Because ABCG1 was shown to regulate sterol transport from cells, multiple groups undertook studies in mice exploring the role of ABCG1 in atherosclerotic lesion development. Despite predictions from the in vitro studies that ABCG1 overexpression would prevent atherosclerotic lesions and conversely that ABCG1 deficiency would increase lesions, the results thus far have been contradictory. While some studies show the expected inverse correlation between ABCG1 protein levels and atherosclerotic lesions, other studies report fewer lesions in Abcg1 knockout mice or an increased accumulation of tissue lipid in ABCG1 overexpressing mice on a high fat diet (Burgess BL, et al. manuscript submitted to J. Lipid Res). Taken together these studies suggest that while ABCG1 is undoubtedly capable of facilitating the export of lipids from cultured cells, the in vivo role of ABCG1 is not entirely understood.

1.4.6. Human disease and Abcg1

There are currently no known diseases with well established genetic linkage to Abcg1. However, because of its position on chromosome 21, Abcg1 is expected to be overexpressed by approximately 1.4-2-fold in with the inheritance of a third copy of chromosome 21 in DS. Interestingly, individuals with DS do show some evidence of dysregulated lipid metabolism, although it should be noted that many of these studies have used very small cohorts with questionable controls. First, trisomic fetuses are reported to be hypercholesterolemic in utero. Secondly, young children with DS are reported to be 23 times more likely to develop cholelithiasis, which are biliary gall-stones associated with cholesterol-saturated bile and high serum cholesterol levels. Third, several groups have measured serum lipids in adults with DS and report that trisomic adults have atherogenic serum lipids, yet paradoxically, appear to be protected from atherosclerosis when compared to similarly institutionalized mentally-retarded controls. Although not conclusive, these results suggest that DS may be associated with aberrations in cholesterol homeostasis and that genes on chromosome 21 such as ABCG1 may contribute to these findings.
1.5. AD mouse models

1.5.1. Modeling AD: Amyloid vs Tau

Over 100 separate animal murine models for studying AD have been reported. These animal models group loosely into two classes, first, tau based models that overexpress tau, or express mutant tau in an attempt to induce AD through tau and tangle pathologies, and second, APP-related models that attempt to induce AD-like pathologies through the formation of Aβ or amyloid deposits. While tau and APP techniques have been successful at producing NFTs and amyloid plaques respectively, neither technique has recapitulated both aspects of pathology without co-expressing mutant forms of tau and APP in the same mouse. A major obstacle to creating a complete model of AD may result from the use of mice as a model species. For instance, humans expressing APP at 1.5-fold normal levels develop autossomal dominant AD that is 100% penetrant by midlife, whereas mice need to overexpress APP more than 8-fold in order to develop amyloid pathology. While no mouse model of AD exists that fully recapitulates AD neuropathology, APP-related amyloid models of AD provide the best in vivo available system for testing treatments based on the amyloid hypothesis and recapitulate amyloid plaques, cerebral inflammation and cognitive deficits consistent with AD.

1.5.2. The PDAPP Mouse model of AD

The PDAPP mouse model of AD is a well-established model of amyloid formation. PDAPP mice express an APP minigene containing introns 7, 8 that permits alternative splicing to produce all the three major human APP isoforms, APP 695, 751 and 770. In the PDAPP mouse all isoforms contain the FAD associated APP V717F "Indiana" mutation that increases the production of Aβ42 by γ-secretase. The APP minigene is expressed under the PDGF-β promoter that drives high expression in hippocampal neurons and cortex. By 12 months of age, PDAPP mice develop amyloid plaques associated with dystrophic neurites and GFAP-positive glial cells that are similar to human pathology, and developed age-related deficits in
learning and memory\textsuperscript{137}. Unlike human AD, however, no significant neuronal loss has been observed in PDAPP mice\textsuperscript{136}. PDAPP mice expressing humanized apoE3 or apoE4 alleles (ie replacement of the murine apoE gene with either human apoE3 or apoE4) confirm that apoE4 accelerates amyloid formation, which is consistent with human studies\textsuperscript{138}. The PDAPP mouse is well suited for these experiments as it is well-established model for studies of cholesterol metabolism and AD and recapitulates multiple aspects of AD including learning and memory deficits, parenchymal amyloid, intracellular amyloid and cerebral inflammation.

1.6. Research Hypothesis and Rationale

1.6.1. Rationale

The amyloid cascade hypothesis of AD asserts that APP processing into Aβ peptides and the accumulation of soluble and fibrillar Aβ in the brain, either by increased production or decreased elimination, are the main drivers of AD neuropathology\textsuperscript{32}. It has been shown that cholesterol levels of the plasma membrane play an important role in Aβ production by modulating the proteolytic processing of APP by secretases\textsuperscript{91,9247}. Thus, because ABCG1 is an important regulator of cholesterol levels and distribution within cells and tissues\textsuperscript{107,116,117,118}, ABCG1 may influence the production of Aβ. Furthermore, individuals with DS overexpress \textit{Abcg1} and \textit{APP} on chromosome 21\textsuperscript{126,127,139} and may accumulate amyloid pathology more rapidly than individuals that overexpress \textit{APP} alone\textsuperscript{99,100}. Thus, the main goal of this thesis is to evaluate the role of ABCG1 overexpression on Aβ production and amyloid formation. Furthermore, because little is known about the role of ABCG1 in the brain in vivo, this thesis has a secondary focus on the role of ABCG1 in mediating cholesterol homeostasis in the brain.

1.6.2. Experimental Hypothesis and Specific Aims

The hypothesis of this thesis is that ABCG1 influences Aβ production and amyloid formation. Specifically, we hypothesize that ABCG1 overexpression will promote amyloid formation by facilitating the production of Aβ peptides in a cell model and from neurons in vivo.
Specific Aims:

1. To determine if ABCG1 over-expression influences Aβ production in vitro

2. To characterize Abcg1 expression patterns in a novel murine model of Abcg1 overexpression

3. To determine the impact of ABCG1 overexpression on brain lipid metabolism in ABCG1 transgenic mice.

4. To establish if ABCG1 overexpression or deficiency influences amyloid neuropathology in a mouse model of amyloid formation.
2. CHAPTER 2: THE ROLE OF ABCG1 IN BRAIN AND AD

2.1. EXPERIMENTAL REAGENTS AND PROCEDURES

2.1.1. Transgenic Animals

2.1.1.1. Generation of ABCG1 BAC Transgenic Mice

ABCG1 bacterial artificial chromosome (BAC) transgenic mice were generated using the 141 kb BAC CTD-201013 (Children's Hospital Oakland Research Institute). The BAC insert contained the entire human Abcg1 genomic sequence plus 30.6 kb 5' and 13.1 kb of 3' flanking sequences. The BAC was purified through pulse-field gradient gels and the presence of all 23 exons was confirmed by PCR. The validated BAC was microinjected into F1 C57Bl/6/CBA murine oocytes, and founder animals were identified by human specific PCR. The stable integration of all 23 exons was verified. Founders were backcrossed onto a pure C57Bl/6 genetic background for at least 5 generations before being used for experiments.

2.1.1.2. PDAPP Mice

PDAPP mice were generously donated by Dr. Ronald DeMattos, Lilly Research Laboratories, Indianapolis, Indiana USA. PDAPP mice express a human APP mini-gene under the control of the PDGF-ß promoter that promotes expression of the transgene in hippocampal and cortical neurons \(^{140}\). The APP mini-gene encodes the FAD associated Indiana mutation (V717F) in addition to APP introns 6-8 to produce APP isoforms 695, 751 and 770 through alternative splicing. PDAPP mice are maintained by brother-sister matings as transgenic homozygotes on a mixed genetic background derived from C57/Bl6, DBA and Swiss-Webster origins.

2.1.1.3. Abcg1-deficient mice:

Abcg1-deficient mice were obtained from Deltagen Inc. The targeting vector used to generate these mice contained 7 kb of 5' and 1.4 kb of 3' murine genomic DNA flanking a 7 kb
Internal Ribosome Entry Site-LacZ-Neo-pA cassette that places the β-galactosidase gene under the control of endogenous Abcg1 regulatory elements. Homologous recombination results in the deletion of 7 amino acids (GPSGAGK) within the Walker A motif in exon 3 of the murine Abcg1 gene to ablate function of the transporter. Chimeric animals were generated using embryonic stem cells derived from the 129/OlaHsd genetic background, and were backcrossed to C57/Bi6 mice for at least 7 generations before use.

2.1.1.4. Generation of Experimental Cohorts

Heterozygous ABCG1 BAC transgenic males were crossed to PDAPP APP homozygous females to produce (ABCG1 BAC+ / APP+) and (ABCG1 wt, APP-) progeny. Female progeny were sacrificed at wean to generate male cohorts for aging experiments.

2.1.1.5. Diet

Animals were maintained on a standard chow diet (PMI LabDiet 5010, containing 24% protein, 5.1% fat, and 0.03% cholesterol)

2.1.1.6. Sacrifice and Tissue Preparation

Animals were anesthetized with 1-1.5mL Avertin™ administered intraperitonealy and prepared according to manufacturer’s instructions. Anesthetized mice were perfused transcardially with PBS containing 2500U/mL heparin at 5-10 mL/min for 7 min. Whole tissues were taken or brains were dissected into regions on ice before snap-freezing on dry ice for storage at -80°C or immersion fixation in 10% formalin in PBS and stored at 4°C.

All procedures involving experimental animals were performed in accordance with protocols from the Canadian Council of Animal Care and the University of British Columbia Committee on Animal Care. See Appendix 1.
2.1.2. Cell Culture

2.1.2.1. HEK 293 Cells

In vitro assays were performed with HEK 293 cells stably transfected with human APP695 Swe (KM670/671NL) that were generously donated by Dr. Weihong Song (University of British Columbia, Vancouver, Canada). HEK 293 cells were chosen for these experiments because they are yield high transfection efficiencies and exhibit α,β and γ secretase activities required to cleave APP into Aβ peptides. HEK 293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat inactivated fetal bovine serum (FBS), 4.5g/L glucose, 2mM L-glutamine, Penicillin (100u/mL) and Streptomycin (100mg/mL) and 200ug/mL G418 (All from Invitrogen) to maintain expression of the APP transgene.

Transfection:

HEK:APP695Swe cells were seeded in 10cm plates at 50% confluency 24h prior to transfection. For transfection, cells were incubated with 25uL FuGENE transfection reagent (Roche) and either 8ug empty vector pFRT (Invitrogen) or pFRT vector containing an Abcg1 cDNA (IMAGE Consortium) containing exons 5,7,11-23, as described[106]. Cells were transfected for 24h, then media was changed to regular growth media for 6h before use. Transfection efficiency was estimated by adding 1ug of eGFP cDNA to the transfection mixture and visualizing GFP fluorescence by inverted microscopy. Transfection efficiency was estimated to be 30-50%.

2.1.2.2. Primary Astrocyte Culture

Primary astrocytes were obtained from 1-2 day post-natal pups. Pups were sacrificed by cervical dissection and brains were removed and placed in cold Hanks Balanced Salt Solution (HBSS) adjusted to 6g/L glucose and 10mM HEPES buffer. Using a dissecting microscope, cortices and hippocampi were isolated, meninges were removed, and brains were triturated by shearing through a 5mL sterile tissue culture pipette. Triturated brain samples
were spun at 1000RCF for 5min, supernatant was discarded and homogenized tissue samples were resuspended in 1 mL of Growth Media (DMEM:F12 containing 10% FBS, 2mM L-Glutamine, Penicillin (100u/mL) and Streptomycin (100mg/mL) all reagents from Invitrogen)). The cell suspensions each from individual pup were added to one T75 tissue culture flask containing 19mL Growth Media for maintenance. Media was changed every 3-5 days until cells were fully confluent, which occurred after approximately 12-14 days in vitro.

2.1.2.3. Primary Neuron Culture

Primary cortical neurons were prepared from E16 or E17 murine embryos. To harvest neurons, embryonic brains were removed and placed in cold HBSS containing 6g/L Glucose, 50mM HEPES. Using a dissecting microscope, cortices were isolated, stripped of meninges and minced using forceps. To free neurons from the extracellular matrix, minced cortices were incubated 3-10min in 0.05% Trypsin at 37°C. Trypsinization was stopped with addition of 2 volumes of Stop Media (Neurobasal media, 10% FBS, 0.5mM L-Glutamine, 1xB27 Supplement, Penicillin (100u/mL) and Streptomycin (100mg/ml, all reagents obtained from Invitrogen) then cells were triturated gently through a 5mL sterile tissue culture pipette and spun 3 min at 1000RFC to collect cells. Crude tissue pellets were resuspended Neuron Growth Media (NGM): (Neurobasal Media, 0.5mM L-Glutamine, 1xB27 Supplement, 1%
Penicillin/Streptomycin (Invitrogen)) and were gently triturated through fire-polished glass pipettes to generate a single cell suspension. Crude cellular debris was allowed to settle for 1min before transferring the cell-containing supernatant transferred to a new tube. Neurons were seeded at a density of one mouse per 24 well plate that had previously been coated overnight with 0.1mg/mL Poly-D-Lysine to facilitate cell adhesion. Neurons were maintained in 800μL/well of NGM that was ½ changed with fresh NGM every 4 days for 14 days in vitro prior to experiments to allow the extension of neurites.
2.1.3. Protein Analysis

2.1.3.1. Sample Preparation

To prepare crude cell or tissue lysates, starting material was homogenized in at least 10 volumes of RIPA Lysis Buffer (20mM tris pH 7.5, 5mM EDTA, 50mM NaCl, 10mM Na Pyrophosphate, 50mM NaF, 1% NP40 alternative, 1x complete protease inhibitor (Roche). Homogenates were sonicated on ice for 15s and centrifuged at 21000 RCF 5min to remove cellular debris.

2.1.3.2. Membrane Fraction Preparation

To improve detection of ABCG1 protein in mouse tissue, crude membrane fractions were purified from murine tissue to eliminate cytosolic proteins prior to Western blotting\textsuperscript{127}. First, 100mg of tissue was homogenized in 1250\mu L Low-ionic Strength Buffer (50mM mannitol, 2mM EDTA, 50mM Tris-HCL pH7.6, 1x Complete Protease Inhibitor (Roche)), centrifuged at 500g for 5min to pellet nuclei and debris, followed by collection of the supernatant. To obtain the purified membrane fraction, 800\mu L of supernatant was layered on a 500\mu L mannitol cushion (300mM mannitol, 2mM EDTA, 50mM Tris-HCL pH7.6) and centrifuged at 100,000 RCF in a fixed angle rotor TLA 120.2 (Beckman) for 45min. The supernatant was discarded and the membrane-fraction pellet was re-suspended with 200\mu L of Low-ionic Strength Buffer supplemented with 1% SDS. The resuspended membrane fractions were sonicated for 15s to shear DNA that could interfere with subsequent SDS-PAGE procedures.

2.1.3.3. Protein Determination assay

The protein content of tissue and cell lysates was determined by DC Protein assay (BioRad) on 0.5-2\mu L of sample containing 0.25-12\mu g protein and was compared to a standard curve of known delipidated-BSA concentrations.
2.1.3.4. SDS-Polyacrylamide Gel Electrophoresis (SDS PAGE)

To facilitate immunodetection of proteins, protein samples were separated by size using SDS-PAGE and transferred to immobilized membranes.

Typical running conditions were as follows.

To prepare samples for SDS-PAGE, the protein content of sample lysates was determined by protein assay and diluted with RIPA buffer to contain equal protein concentrations in equal volumes. The normalized samples were mixed 5:1 with loading buffer concentrate containing 200 mM Tris/HCl pH 6.8, 20% β-Mercaptoethanol, 5% SDS, 20% Glycerol, Bromphenol Blue. SDS and β-mercaptoethanol were included to ensure complete solubilization and saturation of the protein with SDS and reduction of disulphide bonds. Once mixed, samples were heated at 94°C for 5 minutes to ensure hydrolysis of DNA and ensure complete denaturation of all proteins.

Protein samples were loaded into polyacrylamide gels either 0.75 mm or 1.5 mm thick. Gels contained 3 volumes separating gel to 1 volume stacking gel to ensure good resolution. Separating gels contained 10% acrylamide/bis 375 mM Tris pH 8.8, 0.01% SDS, 0.005% ammonium peroxydisulphate (APS), pH 8.8. Stacking gels contained 3% bis-acrylamide, 0.125 mM Tris pH 6.8, 0.02% SDS, 0.01% APS. Polymerization was initiated with TEMED (Biorad).

Anode and cathode buffer reservoirs were filled with Running Buffer containing 6 mM Tris pH 8.4, 4.7 mM glycine, 1 μM SDS. Gels were run 1-1.5 h at 100 V at room temperature.

Tris-Tricine Gels

To resolve C83 and C99 CTFs, a Tris-Tricine buffer system was used to separate proteins on step gradient gels that maximized resolution of the CTFs and APP holoprotein. Samples were prepared by mixing 4:1 with Tris-Tricine loading buffer concentrate containing
(100mM Tris-HCl pH 6.8, 30% glycerol, 8% SDS, 0.02% Commissie Blue G-250, 3% DTT).
Step gradient gels consisted of 3 layers: stacking gel (4% acrylamide, 0.75mM Tris pH 8.5, 0.02% APS), middle separating layer (10% acrylamide, 1M Tris pH 8.45, 13% glycerol, 0.02% APS) and bottom separating layer (17% acrylamide, 1M Tris pH8.45, 13% glycerol, 0.02% APS) polymerized with TEMED (BioRad). Gels were poured with a ratio of 1:1:3 (4%:10%:17%)(v/v/v). The cathode chamber was filled with Cathode Running buffer (100mM Tris-base pH 8.5, 100mM tricine, 0.1% SDS) and the anode buffer reservoir was filled with anode buffer (200mM Tris-base pH 8.9). Tris-Tricine gels were run at 110V for 5-6h at room temperature and transferred to nitrocellulose membranes.

Transfer:

To transfer proteins from polyacrylamide gels to immobilized nitrocellulose or polyvinylidene fluoride membranes for western blotting, gels were sandwiched with membrane and blotting paper and immersed in Transfer Buffer containing 25mM Tris pH 8.4, 190mM glycine,10% methanol. Gels were transferred at 24-36V for 3-24h at 4°C.

2.1.3.5. Western blotting

The following Western blot protocol was used to detect proteins except where noted. Membranes were blocked in 5% skim-milk in Phosphate Buffered Saline (PBS) containing (10mM Na₂HPO₄, 1.4mM KH₂PO₄, 137mM NaCl, 3mM KCl) for 1h to reduce non-specific antibody binding in subsequent steps. All incubations were performed with agitation. Primary antibodies were prepared in 5% skim milk powder in PBS. Antibody dilution and incubation time varied for each antibody as described below. Following incubation with primary antibodies, the membranes were washed 3x 10 minutes with PBS supplemented with 0.05% Tween-20 detergent (PBS-T). Secondary detection was performed using HRP-conjugated antibodies specific to the host-species of the primary antibody, diluted 1:2000 in 5% skim milk powder in PBS and incubated for 30min to 2h. Following the binding of secondary antibodies,
membranes were washed 3x 10min in PBS-T, immersed in Enhanced-Chemiluminescent reagent (Amersham) for 1 min and exposed to photographic film.

ABCG1:

Total cell lysates were used to detect ABCG1 in HEK cells, whereas crude membrane fractions were used to detect ABCG1 in murine tissues. Samples were diluted with loading dye and heated for 5min at 55°C. Western blot conditions were as previously listed, except PBS and PBS-T were replaced by Tris-Buffered-Saline (0.3% Tris pH 7.4, 0.8% NaCl, 0.02%KCl)(TBS) and TBS-T (TBS, 0.05%Tween-20). The primary antibody was rabbit anti-ABCG1 NB400-132 (Novus) diluted 1:750. Secondary detection was by goat anti-rabbit-HRP conjugated (ImmunoLabs) diluted 1:2000 in 1% skim-milk TBS and incubated for 2h.

GAPDH

We determined GAPDH levels to control for protein loading. The primary antibody was mouse monoclonal mAB374 (Chemicon) diluted 1:5000 for 30min. Secondary antibody conditions were 1:2000 goat anti-mouse-HRP incubated for 45min.

APP-CTF

For experiments involving the detection of APP CTFs, nitrocellulose membranes were cut into holoprotein and CTF containing segments at a position corresponding to approximately 60kDa. The holoprotein membrane segment was incubated with rabbit anti-APP-C-terminal antibody (Sigma-Aldrich) diluted 1:5000k for 1h and with secondary bovine anti-rabbit-HRP diluted 1:2000 for 45min. The CTF containing membrane was incubated in rabbit anti-APP-C terminal antibody (Sigma) diluted 1:5000 overnight. Secondary antibody conditions were goat anti-rabbit-HRP diluted 1:2000 for 2h.
APP

To detect APP, the primary antibody was mouse monoclonal 6E10 (SantaCruz Biotech) diluted 1:1000 for 1h. Secondary antibody conditions were goat anti-mouse-HRP diluted 1:2000 and incubated for 1h.

ApoE

To detect apoE protein, proteins were transferred to Immobilon-P PVDF membranes (Millipore). The primary antibody was polyclonal rabbit anti-apoE M-20 (SantaCruz Biotech) diluted 1:750 for 2h. Secondary antibody conditions were bovine anti-rabbit-HRP diluted 1:2000 and incubated for 1hr.

Quantitation

Western blot data was quantitated using densitometry. Photographic film was scanned into TIFF format at 600dpi resolution and pixel counts were determined using ImageJ (NIH) software.

2.1.4. Messenger RNA Analysis

2.1.4.1. mRNA Extraction

Total mRNA content was extracted using Trizol (Invitrogen) according to the manufacturer’s protocol from cells or snap-frozen tissue that was previously stored at -80°C. Extracted mRNA was and stored at -80°C for fewer than 3 months prior to use. To reduce DNA contamination, samples were treated with DNAsel prior to cDNA synthesis.

2.1.4.2. QRT-PCR

The level of mRNA was determined using quantitative real-time polymerase chain reaction (QRT-PCR). Prior to QRT-PCR, mRNA transcripts were converted to cDNA using oligo-dT primers and Taqman Reverse transcription reagents (Applied Biosystems). Primers
were designed using PrimerExpress software (Applied Biosystems) and spanned exons 22-23 of human \textit{Abcg1}. Sequences are: Human \textit{Abcg1} forward (5' ACACCATCCCACGTACCTA 3') and reverse (5' GATGACCCCTTCGAACCCA 3') and murine \textit{Abcg1} forward (5' CAACGTGGATGAGGTTGAGA 3') and reverse (5' CTGGGCCTCTGTGAAGTTGT 3'), and murine \(\beta\)-actin forward (5' ACGGCCAGGTCATCACTATTG 3') and reverse (5' CAAGAAGGAAGGCTGGAAAAG 3').

QRT-PCR was done with Sybr green reagents (Applied Biosystems) on an ABI 7000. Cycling conditions were 50°C for 2 min, 95°C for 10 min, then 40 cycles at 95°C for 15 sec and 60°C for 1 min, followed by dissociation at 95°C for 15 sec, 60°C for 20 sec, and 95°C for 15 sec. Each sample was assayed in triplicate, normalized to \(\beta\)-actin to control for RNA loading and analysed with 7000 system SDS software v1.2 (Applied Biosystems) using the relative standard curve method.

2.1.5. Lipid Analysis

2.1.5.1. Total Lipid Analysis

Lipid Extraction:

To determine total lipid content of brain tissue, 25mg of tissue was homogenized in 0.9% saline and protein content was determined by Lowy assay (BioRad). Brain homogenates were mixed with 2:1 chloroform:methanol mixture to obtain a final dilution of 6:3:1.25 chloroform:methanol:saline containing betulin as an internal standard. Samples were spun at 400RCF for 5 minutes to separate the aqueous and organic phases. The organic layer was isolated and an equal volume of chloroform was added back to the tissue homogenate to re-extract lipids from the aqueous phase. Samples were vortexed and centrifuged as before and the organic layers were pooled. The pooled organic fractions containing total lipid extracts were evaporated under nitrogen and solubilized in chloroform-methanol-acetone-hexane (2.0:3.0:0.5:0.5) for storage at -20C until analysis.
Lipid Measurements

Lipid analysis was performed in collaboration with Dr. Sheila Innis (University of British Columbia, Vancouver) as described\textsuperscript{141}. Separation of polar and non-polar lipids, including individual classes, was achieved using a HPLC (Waters 2690 Alliance HPLC, (Milford MA), equipped with an auto-sampler and column heater. The sample chamber was kept at 18°C and the column heater at 35°C. The column was a Waters YMC-Pack Diol 120NP, 25 cm 4.6 mm id, 5 m particle size and 12 nm pore size. Using a quaternary solvent system of hexane-petroleum ether, 97:3 (v,v); methanol-triethylamine-acetic acid, 765:15:13 (v/v/v); acetone-triethylamine-acetic acid, 765:15:13 (v/v/v); isopropanol-acetic acid, 800:40 (v/v) in a linear gradient with a flow rate of 2 mL/min. The column eluant was split 10:90 to an ELSD (Alltech, model 2000; Mandel Scientific, Guelph, Canada) and a fraction collector (Gilson FC204, Mandel Scientific). ELSD detection and quantitation of the separated lipid classes was performed with a nitrogen flow rate of 1.8 ml/min, a drift tube temperature of 60°C. Calibration curves to determine the linear range of the analysis were established using authentic standards for each lipid class and samples were quantified using the external standard method.

2.1.5.2. Cholesterol metabolites

Lipids were extracted from tissues by a modified Folch procedure. Briefly, dried tissue samples were submerged in chloroform:methanol (2:1) overnight in a capped dram vial. The following day, solvent volume was adjusted to 20X the dry weight, and 0.25x volumes of 0.9% NaCl was added, mixed vigorously for 1 min, and equilibrated by shaking for 2h at RT. Samples were centrifuged at 2000 rpm for 5 min to separate phases. The upper aqueous phase was removed, and lipids were dried under an N\textsubscript{2} stream. Sterol levels were determined after derivatization to the corresponding trimethylsilyl-ethers by gas chromatography-flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS) as reported previously\textsuperscript{142}.
2.1.5.3. Cholesterol efflux

The mass of cholesterol transferred to apolipoprotein, or lipoprotein acceptors was used to assess cholesterol transporter activity as described \(^{143}\).

HEK cells:

Cells were seeded in 24 well plates at 250,000 cells / well for efflux. Cellular cholesterol was labeled with 1\(\mu\)Ci/mL of \(\text{H}^3\) cholesterol for 24h during transfection in growth media. Transfected and labeled cells were washed once with Efflux Media (DMEM, 0.2% BSA, 2mM L-Glutamine) and incubated for 4h efflux in either efflux media or efflux media containing human HDL2/3 (25\(\mu\)g/mL HDL protein).

Primary Astrocytes:

Astrocytes were seeded in 24 well plates and allowed to adhere overnight. To prepare efflux Loading Media, Acetylated-LDL (Intracel) was incubated with \(\text{H}^3\)cholesterol (New England Nuclear) for 30min at 37°C before dilution in DMEM, 1% FBS, 2mM L-glutamine, 100U/ml penicillin, 100mg/mL streptomycin to obtain a final concentration of 40\(\mu\)g/mL AcLDL protein and 1\(\mu\)Ci/mL \(\text{H}^3\)cholesterol. Cells were incubated in Loading Media for 24h, washed once in efflux media and equilibrated 4h in Efflux Media to internalize membrane bound lipoproteins and distribute radiolabeled cholesterol within the cell. Cells were washed once in efflux media prior to 4h efflux in 250\(\mu\)L Efflux Media supplemented with either nothing, 15\(\mu\)g/mL delipidated human apoAI (Calbiochem), 15\(\mu\)g/mL delipidated human apoE3 (Calbiochem) or human HDL\(_{2/3}\) containing 25\(\mu\)g/mL HDL protein.

Quantification

After incubation with efflux media, the cell media was collected and spun for 5 min at 21000 RCF to remove cells and was mixed with two volumes of OptiPhase SuperMix.
scintillation fluid (PerkinElmer). Cells were lysed with 100µL 0.1% NaOH, 0.2% SDS and mixed with 3 volumes of scintillation fluid. The H³ content of cells and media were determined by scintillation counting to calculate efflux.

Efflux = (media counts) / (media counts + cell counts).

2.1.6. LacZ staining

To prepare tissue for Lac-Z staining, wildtype and Abcg1 hemizygous mice were perfused with PBS. Brains were immersion fixed in 2% paraformaldehyde PBS for 24h and cryoprotected in 30% sucrose in PBS at 4°C. Frozen coronal sections were prepared on a cryostat and mounted on Superfrost Plus Slides (Fisher). LacZ staining was performed in the presence of 1mg/mL X-gal. Sections were counterstained with Neutral Red and dehydrated prior to being coverslipped.

2.1.7. Aβ Measurements

2.1.7.1. Aβ Secretion Assays

The secretion of Aβ from HEK:APPSwe cells was measured from transiently transfected cells seeded in 10cm plates. To measure Aβ secretion, 10 ml of fresh Growth Media was added to cells for 6h, then removed and spun at 21000 RCF for 5 min to pellet contaminating cells. The cell-free media was supplemented with complete protease inhibitor (Roche) to reduce degradation of Aβ and was frozen at -80°C until further use. Plated cells were scraped into 1mL of PBS, spun at 21000 RCF to remove supernatant, then homogenized in 200µL RIPA buffer as previously described. The total protein content of RIPA lysates was determined by Lowry assay. To determine the Aβ40 and Aβ42 content of conditioned media, media samples were diluted 5-10x and measured by ELISA (Biosource) specific to human Aβ40 or 42 as per manufacturers instructions. Raw Aβ values were normalized to total plate protein levels to account for plate to plate differences in seeding density and differences in cell-survival between ABCG1 and empty vector transfected cells.
2.1.7.2. Murine Aβ measurements

Endogenous murine Aβ levels were quantified using 75 μg of protein from total brain homogenates by Dr. Ronald DeMattos of Lilly Research Laboratories, Indianapolis. The coating antibody used was R163 for Aβ40 (a generous gift from Dr P.D. Mehta, New York Institute for Basic Research, Staten Island, New York). Plates were coated overnight at 4°C and then blocked with PBS containing 0.1% BSA for 2h at room temperature. Following 5 washes with TBS-T (Tris-buffered saline containing 0.1% Tween-20), samples were incubated for 2h with biotinylated 4G8 (Signet Laboratories Inc) for 1h with agitation. The plates were washed 5X and streptavidin-alkaline phosphatase complex was added for 1h. Plates were finally washed 5x with TBS-T and once with water and AttoPhos reagent (Calbiochem) was added for 30-60 min and fluorescence was determined using a Bio-Tek FL600 fluorescence microplate reader. Murine Aβ levels were normalized to total protein as measured by DC Protein Assay (BioRad).

2.1.7.3. Human Aβ measurements

Human Aβ levels in tissues were determined by Dr. Ron DeMattos, Lilly Research Laboratories, Indianapolis as described. Briefly, hippocampal tissue was homogenized in PBS and spun 5 min at 21K RFC to pellet insoluble debris and supernatant was used for soluble Aβ measurements. Cellular debris was resuspended in denaturing buffer containing 5 M guanidine plus protease inhibitors. The extracts were diluted and analyzed in denaturing ELISAs containing a final concentration of 500 mM guanidine for Aβ Total (m266 as capture antibody and biotinylated m3D6 as reporter antibody) or Aβ42 (m21F12 as capture antibody and biotinylated m3D6 as reporter antibody). Aβ40 levels were determined by subtraction.
2.1.8. ApoE ELISA

apoE levels in CSF were determined by ELISA as described\textsuperscript{145}. To prepare ELISA plates, MaxiSorp (Nunc) 96-well plates were coated with capture antibody WUE4 generously provided by Dr. Holtzman (Washington University, St. Louis) diluted at 4mg/mL in Carbonate ELISA Buffer (50mM NaHCO\textsubscript{3} pH 9.6) overnight at 4°C. All incubations were performed with agitation. Plates were washed 5X with PBS supplemented with 0.25% Tween-20 (PBS-T) and blocked with 1% skim milk powder in PBS-T for 1h. Samples and standards were prepared ELISA Buffer (PBS-T, 0.5% BSA). apoE standards were prepared from Swiss-Webster mouse plasma calibrated to contain 61.7μg/mL apoE. The standard curve ranged from 0.8ng/mL to 50ng/mL apoE. Samples and standards were loaded onto the plate and incubated overnight at 4°C. To wash the plate, wells were rinsed 5x with PBS-T. The detection antibody was goat polyclonal anti-apoE (Calbiochem cat#178479) diluted 1:6000 in ELISA Buffer and was incubated for 1.5h at room temperature. The plate was then washed 5x with PBS-T prior to incubation with biotinylated anti-Goat (Vector BA-9500) diluted 1:50,000 in ELISA Buffer for 1.5h. The plate was washed again 8x with PBS-T prior to incubation with streptavidin-poly-HRP (Pierce) diluted 1:6000 in ELISA buffer for 1.5h. To quantify apoE, plates were washed 8x with PBS-T prior to the addition of ELISA-TMB (Sigma-Aldrich) until adequate development of the coloured product. The ELISA reaction was stopped with the addition of 1N HCL. Absorbance at 450nM was measured with a MultiScan microplate reader (Fischer).

2.1.9. Statistical Analyses:

All p-values reported are derived from two-tailed Student's t-test analysed by Prism GraphPad software 3.0.4. Error bars reflect standard deviation.
2.2. RESULTS

2.2.1. ABCG1 overexpression in vitro

To determine the impact of ABCG1 overexpression on APP processing, we first conducted experiments in human embryonic kidney (HEK) 293 cells stably expressing human APP695Swe (HEK:APP cells) that were transiently transfected with a human Abcg1 cDNA or empty vector as a negative control. Although HEK cells are non-neuronal in origin, they do express all of the components of α, β and γ secretase required to process APP into Aβ and have been used extensively to study APP metabolism.\(^{146,147}\)

2.2.1.1. ABCG1 overexpression increases cholesterol efflux to HDL

To validate the functionality of the Abcg1 cDNA construct used in our transfection assay, we tested whether transient ABCG1 overexpression increases cholesterol efflux to HDL, which is the best-established assay for ABCG1 enzymatic activity.\(^{107,116,117}\) The level of cholesterol efflux from HEK:APP cells transfected with wildtype ABCG1 was compared to cells transfected with either empty vector, or to ABCG1 containing a S220G mutation (ABCG1-S220G) in a conserved residue in the ATP-binding domain that is expected to eliminate functional activity. HEK:APP cells expressing wild-type ABCG1 exhibited a 21% increase in cholesterol efflux to HDL (p=0.003, N=6 (Figure 2-1A)) compared to either empty vector or ABCG1-S220G, which were indistinguishable from each other. These results confirm that ABCG1 functions as a cholesterol transporter in HEK:APP cells and that the S220G variant is functionally inactive with respect to cholesterol efflux activity.

2.2.1.2. ABCG1 overexpression promotes Aβ secretion in vitro

Having validated the HEK:APP model system, we then assessed the impact of ABCG1 overexpression on Aβ production. HEK:APP cells were transfected with empty vector, functionally inactive S220G ABCG1 or wild-type ABCG1, and conditioned media was collected
Figure 2-1: ABCG1 overexpression increases cholesterol efflux to HDL and increases the secretion of Aβ40 and Aβ42. (A) HEK-APP cells were stably transfected with empty vector, ABCG1 or ABCG1 harbouring an S220G mutation in the catalytic domain. Expression of ABCG1 enhanced the efflux of cholesterol to HDL over 5 hours, compared to either empty vector or ABCG1 S220G. Data are representative of 2 independent experiments done in triplicate. (B) The overexpression of ABCG1 in HEK-APP cells increases the secretion of Aβ40 (N=2) and (C) Aβ42 into cell culture medium over a 6 hour period (N=2). This effect requires the transporter activity of ABCG1. (D) Western blot analysis confirms overexpression of ABCG1 in HEK-APP cells at the protein level. ns=p>0.05, *p<0.05, **p<0.01
to measure Aβ secretion from cells. Compared to empty vector, wild-type ABCG1 increased the levels of secreted Aβ40 by 60% (p<0.05, N=2) (Figure 2-1B) and Aβ42 by 28% (p<0.05, N=2) (Figure 2-1C). Aβ secretion from ABCG1-S220G transfected cells was similar to empty vector. These results demonstrate that ABCG1 increases Aβ secretion from cells, and that the functional activity of ABCG1 as a cholesterol transporter is required for this effect.

2.2.1.3. ABCG1 overexpression increases APP processing in vitro

The level of Aβ secreted from HEK:APP cells was increased by the presence of functional ABCG1 however, additional experiments were required to determine the underlying mechanism. As changes in membrane cholesterol levels are reported to influence Aβ production by modulating the proteolytic processing of APP by secretases, we measured APP processing intermediates that reflect the activity of α and β secretase.

During the proteolytic processing of APP into Aβ, C-terminal processing intermediates of either 83 or 99 amino-acids in length are created at the plasma membrane through proteolysis by α or β secretase respectively (Figure 1-1)\textsuperscript{148,149}. The membrane bound CTFs are internalized where they can be further cleaved by γ-secretase. C83 is cleaved by γ-secretase into a harmless product known as p3. Alternatively, C99 is processed into Aβ. Because C99 is the direct precursor to Aβ, the levels of intracellular C99 fragments are the gold standard to estimate the relative rate of Aβ production\textsuperscript{146,147}.

To determine if elevated APP processing accounted for the increased Aβ secreted from ABCG1 transfected cells, C83 and C99 levels were measured by Western blot. Western blots were prepared from RIPA total cell lysates from HEK:APP cells transfected with ABCG1 or empty vector, and were immunoblotted with antibodies specific to the last 19 amino-acids of the C-terminus of APP to detect C83 and C99 (Figure 2A). Compared to vector-transfected controls, C83 levels in HEK:APP cells transfected with ABCG1 were increased 2.9 +/-0.4-fold (p=0.014, N=3) (Figure 2-2B) and C99 levels were increased 4.5 +/-0.7-fold (p<0.006, N=3) (Figure 2-2D).
Figure 2-2: ABCG1 overexpression increases cellular APP levels and increases the processing of APP into CTFs. (A) Western blots of HEK-APP cell-homogenates previously transfected with either empty vector or ABCG1 were probed with antibodies specific to the C-terminus of APP to detect full-length unprocessed APP or CTFs. (B,D) C83 and C99 levels are increased in ABCG1 transfected cells. (C,E) When normalized to total APP levels, C83/APP and C99/APP are significantly increased compared to empty vector.
Total unprocessed APP holoprotein in ABCG1 transfected cells was non-significantly increased by 1.6-fold (p=0.094, N=3) compared to cells transfected with empty vector.

To determine whether the increase in CTF levels reflected an increase in APP processing or could reflect the trend toward elevated APP levels, C83 and C99 levels were expressed as a ratio to total APP. We found that compared to empty vector, ABCG1 overexpression increased the C83 to APP ratio by 1.7 +/-0.16-fold (p<0.014, N=3) (Figure 2-2C) and the ratio of C99 to APP by 2.8 +/-0.13-fold (p<0.0002, N=3) (Figure 2-2D). The finding that C99 levels are increased, even when corrected for total APP levels, confirms that ABCG1 overexpression increases Aβ production under these experimental conditions.

2.2.2. ABCG1 expression in brain

In individuals with AD, neuropathology is first detected in the hippocampus and entorhinal cortex. Although the precise mechanism behind amyloid and NFT formation in sporadic AD is not completely understood, the amyloid cascade hypothesis suggests that the production and secretion of Aβ peptides by neurons in these regions is a crucial step in AD pathogenesis. Aβ peptides are produced by several cell types in the body including neurons and platelets, and neurons are reported to produce the majority of Aβ within the central nervous system. Because our in vitro results show that ABCG1 overexpression increases Aβ production, determining the physiological relevance of this finding requires knowledge of whether ABCG1 is expressed in neurons.

2.2.2.1. Abcgl is highly expressed in hippocampal neurons

The presence of Abcgl mRNA in brain had been reported prior to this study, however the specific regions and cell types that express Abcgl were not well described. To further address the question of Abcgl expression within the brain, we used Abcgl knockout mice that were generated by inserting a β-galactosidase (LacZ) reporter construct into the coding region of Abcgl. Because the LacZ reporter gene is expressed under the control of
endogenous Abcg1 regulatory sequences, Abcg1 expression patterns in the CNS can be accurately determined by staining for β-galactosidase activity. Coronal sections of adult mouse brain hemizygous for Abcg1 were therefore stained with X-gal to visualize Abcg1-expressing cells (Figure 2-3). Comparison of X-gal staining in Abcg1+/− sections (Figure 2-3A,2-3A) to Abcg1 +/+ brains (Figure 2-3B, 2-3D) reveals marked Abcg1 expression in the dentate gyrus and CA1, CA2, and CA3 neuronal layers of the hippocampus, and detectable Abcg1 expression in cortical layers (Figure 2-3F-I). These observations confirm that Abcg1 is indeed expressed in neurons that are highly relevant to AD pathogenesis. Intermediate staining intensity indicative of moderate Abcg1 expression was also observed in striatal and thalamic regions.

Although these results suggest that Abcg1 is predominately expressed in neurons, they do not exclude the possibility that Abcg1 may also be expressed in other cell types in the brain. For example, because Abcg1 is known to be expressed in peripheral macrophages, which are highly related to microglia in the brain, we expected to observe Abcg1 expression in microglia. Abcg1 expression has also been reported in astrocytes. What is clear from the histological results is that expression of Abcg1 is high in neurons under basal conditions, particularly for hippocampal neurons known to be highly relevant for AD. These observations support the hypothesis that ABCG1 could accelerate Aβ production and AD neuropathology as observed in DS, by increasing the level of Aβ production in neurons.

2.2.3. Generation of a novel animal model of ABCG1 overexpression

Our in vitro findings show that ABCG1 overexpression increases the rate of Aβ production from cells. The major caveat of these findings however, is the use of a non-neuronal cell model in which ABCG1 and APP were both highly overexpressed. To confirm our in vitro findings in a physiologically relevant context an ABCG1 overexpressing mouse was therefore generated.
Figure 2-3: *Abcg1* expression in brain. (A) Coronal sections from mice hemizygous for a LacZ reporter gene under the control of *Abcg1* regulatory sequence were stained with X-gal to detect the presence of the reporter and were counterstained with neutral red. *Abcg1* expression is detected in the hippocampus, cortex, striatum and thalamus. (B,F) Wildtype sections show no endogenous lacZ activity. (C) *Abcg1* is highly expressed in neurons of the CA1, CA2 CA3, and Dentate Gyrus of the hippocampus. (E,G,H,I) *Abcg1* expression is enriched in cortical layers.
2.2.3.1. Transgenic strategy

Because we were attempting to recapitulate overexpression of \textit{Abcg1} through an increased gene dose as seen in DS, the transgenic strategy employed needed to overexpress the correct \textit{Abcg1} mRNA isoforms in the correct cell types; information that was not available at the time this model was generated. We therefore selected genomic \textit{Abcg1} maintained in a BAC as a transgene, as this approach has been shown to produce overexpression of many transgenes in a physiologically regulated way that maintains proper cell type and developmental expression patterns \cite{154}. The BAC is essential to this approach as bacterial plasmid vectors cannot maintain sufficiently large inserts to accommodate genomic DNA spanning most eukaryotic genes \cite{154,155}. The ABCG1 BAC insert used here contains a 141kb region of human chromosome 21 that contains all introns and exons of \textit{Abcg1}, 31kb of upstream regulatory sequence containing all known promoter elements and no other known genes.

Purified BAC DNA was injected into mouse oocytes for random integration into the genome. Tail DNA from the resulting progeny were tested by PCR for inheritance of the BAC using human specific primers (data not shown), and 3 transgenic founders from independent injections were verified. After 3 generations of backcrossing to congenic C57/Bl6 mice, the relative levels of transgene expression was measured by QRT-PCR in the brain. Brain was chosen for these experiments as ABCG BAC Tg mice would be used primarily to evaluate the role of ABCG1 on lipid homeostasis and AD neuropathology in the brain. The expression of human \textit{Abcg1} in mice derived from founder 2 was \~73-fold greater than mice derived from founder 3, that showed the lowest level of transgene expression (Figure 2-4). Line 2T was derived from the progeny of founder 2, however these mice lacked human exons 1-4 (data not shown) and expressed human \textit{Abcg1} at low levels. We hypothesize that the line 2T created by the integration of a truncated BAC in founder 2, that segregated from the full-length BAC during backcrossing. Because \textit{Abcg1} expression in line 2 was \~5-fold greater than the next highest expressor, line 2 was chosen for further experiments. The DNA sequence of human \textit{Abcg1}
Figure 2-4: ABCG1 BAC expression in brain of transgenic founders. Expression of the ABCG1 BAC in brain of four independent transgenic strains was compared to select the highest expressing line for further experiments. Human Abcg1 mRNA was determined by QRT-PCR and normalized to the lowest expressing line for comparison.

exons 1-23 in line 2 was validated by PCR to confirm the absence of coding mutations (data not shown).

2.2.3.2. Expression of the Abcg1 transgene is physiologically regulated in vivo

Once the ABCG1 BAC transgenic mice had been generated, transgene expression was characterized to establish whether the ABCG1 BAC Tg mouse was an accurate model of increased ABCG1 gene dose. First we measured mRNA levels in 12 different tissues previously reported to express Abcg1 to determine if Abcg1 was properly expressed across tissue specific axes. The levels of either human or murine ABCGG1 mRNA were determined by QRT-PCR and were normalized first to the levels of β-actin to control for RNA loading and next (in a ratiometric manner) to Abcg1 mRNA in liver for each species (Figure 2-5). Because the method used to quantify mRNA cannot directly compare the amount of human
to mouse mRNA, the results were normalized to an internal reference tissue and expressed as fold-increase. Liver was chosen as our internal reference as \textit{Abcg1} was reported to be expressed in liver and would permit the comparison of our results to previously published data that were also normalized to liver. Relative levels of human and murine mRNA did not significantly differ in 10 of the 12 tissues examined as determined by Student's t-test (Figure 2-5). The two tissues that did show differences between endogenous and transgenic expression were adrenal gland and spleen, where human \textit{Abcg1} expression was 76\% lower than endogenous murine \textit{Abcg1} in adrenal gland (p<0.05) and 75\% lower in spleen (p<0.001)(N=3 human \textit{Abcg1} and N=6 murine \textit{Abcg1} for all levels measured). Human \textit{Abcg1} mRNA in eye was non-significantly increased compared to murine expression. Other than these two tissues, the similarities between relative levels of human and murine \textit{Abcg1} mRNA suggest that the BAC is expressed in a physiologically accurate way. Most importantly for this study, expression of the human \textit{Abcg1} transgene in brain reflected that of endogenous \textit{Abcg1}.

2.2.3.3. Murine and human expression of \textit{Abcg1}

We also compared murine \textit{Abcg1} levels from this experiment with a published report detailing expression of \textit{Abcg1} mRNA in human tissues\textsuperscript{107}. The authors used Northern blots to quantify \textit{Abcg1} mRNA levels, defined liver expression as 100\% and other tissues were expressed as a percentage of liver. Because the mRNA levels in Figure 2-5 are normalized to liver, these murine data can be compared to the human data. Results of the studies are summarized in Table 2-1. These results suggest that patterns of \textit{Abcg1} expression are substantially different between mice and humans.

2.2.3.4. The ABCG1 BAC is expressed in neurons

Because neurons generate the majority of A\textsubscript{\text{3}} peptides in the brain, it was important to determine if the \textit{Abcg1} transgene was expressed murine neurons\textsuperscript{151,152}. To measure mRNA levels in neurons from the BAC transgenic mice, cortical neurons were cultured from ABCG1
Figure 2-5: The tissue-specific pattern of the ABCG1 BAC transgene expression is similar to murine Abcg1. Abcg1 mRNA levels were determined by QRT-PCR, normalized to beta-actin to control for loading and expressed as fold-induction relative to liver for comparison. (A) Murine Abcg1 mRNA was obtained from wildtype male mice and measured using murine specific primers to exon 2 (N=4). (B) Human Abcg1 mRNA was obtained from ABCG1 BAC Tg males and was amplified using human specific primers spanning exons 22-23 (N=3).

BAC Tg and wildtype E16 embyros. Neurons were allowed differentiate for 14 days in vitro before treatment with TO901317, a synthetic LXR ligand known to stimulate expression of genes involved in cholesterol efflux including Abcg1. Neuronal mRNA was harvested after 24 hours of treatment and murine and human Abcg1 mRNA was quantified by QRT-PCR. Compared to vehicle treatment, the levels of murine Abcg1 mRNA were increased 1.41±0.04-
fold (p =0.005, N=4) by treatment with 0.5μM TO901317, or 1.34-fold ± 0.09 (p=0.039 N=4) following treatment with 2μM TO901317 (Figure 2-6). Similarly, the levels of human Abcg1 mRNA were significantly increased relative to vehicle by 1.57± 0.01-fold (p=0.002, N=4) after treatment with 0.5μM TO901317 and by 1.96 ± 0.39-fold (p=0.05, N=4) after treatment with 2μM TO901317 (Figure 2-6). This result shows that both human and murine Abcg1 are expressed in Abcg1 transgenic neurons. Furthermore TO901317 induced similar amounts of

<table>
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</tr>
<tr>
<td>Testes</td>
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</tr>
</tbody>
</table>

* Klucken et al. (2000) PNAS

Table 2-1: Tissue specific levels of Abcg1 mRNA in humans and mice. Human Abcg1 mRNA levels measured by northern blot were obtained from a previously published report. Murine Abcg1 mRNA levels in wildtype mice were determined by QRT-PCR. Both mRNA species are expressed as relative units. Liver is defined as 1 for reference.
human and murine mRNA expression in neurons, suggesting that both the human transgene and murine Abcg1 respond similarly to oxysterol ligands.

2.2.3.5. ABCG1 transgenic mice overexpress ABCG1 protein 3-8 fold in peripheral tissues

Because the correlation coefficient between mRNA and protein levels is less than 0.5, and absolute levels of human Abcg1 mRNA cannot be directly compared to endogenous murine Abcg1 mRNA levels, it is crucial and more relevant to determine the levels of ABCG1 overexpression in the ABCG1 BAC Tg mice at the protein level. Detection of ABCG1 protein in total tissue homogenates proved to be insensitive using all available ABCG1 antibodies (data not shown). We therefore enriched for ABCG1 by preparing crude membrane fractions from
total tissue homogenates over a mannitol gradient, and these membrane fractions were used to prepare Western blots of ABCG1 in tissues. Using this method, we observed that ABCG1 protein was robustly expressed in spleen, liver and lung of ABCG1 BAC Tg mice compared to nontransgenic littermate controls (Figure 2-7A). In these tissues, the level of ABCG1 protein was 3-5 fold greater in ABCG1 BAC Tg mice compared to nontransgenic littermates. Because human and murine ABCG1 are 97% similar at the protein level, the antibody used does not noticeably differentiate between human and murine ABCG1 (data not shown). Thus, the levels of ABCG1 protein shown here reflect the sum of both endogenous murine and transgenic human ABCG1 protein.

2.2.3.6. ABCG1 is overexpressed 3-6-fold in cortex, hippocampus and cerebellum

Because our ABCG1 BAC Tg model would be used to assess amyloid pathology and amyloid develops in a model that develops these deposits in a regional specific manner, we performed ABCG1 Western blots on selected brain regions. Compared to wildtype mice, ABCG1 protein levels in BAC Tg mice were increased 6.0± 0.47-fold (p=0.001, N=3) in cortex, 5.6± 1.5-fold (p=0.038, N=3) in hippocampus and 3.0± 0.48-fold (p=0.025, N=3) in cerebellum (Figure 2-7B). These results confirm that ABCG1 is overexpressed in brain regions relevant to AD in our ABCG1 BAC Tg mice, validating this model as suitable for determining whether physiologically accurate overexpression of ABCG1 is sufficient to accelerate amyloid pathology in vivo.

2.2.3.7. ABCG1 remains overexpressed in PDAPP mice with amyloid

The analyses of ABCG1 protein levels were performed using baseline ABCG1 BAC Tg mice prior to breeding to an animal model of AD. This is important because we had previously observed that the expression of a related ABC transporter, ABCA1, was altered upon crossing to the APP/PS1 mouse model of AD, suggesting that the presence of amyloid deposits may
Figure 2-7: ABCG1 protein is overexpressed in ABCG1 BAC Tg tissues. Crude membrane fractions were prepared from tissues of wildtype or ABCG1 BAC Tg mice for Western blot of ABCG1 and NaKATPase transporter as a loading control. (A) Compared to wildtype mice, ABCG1 is overexpressed 3-5-fold in spleen, lung and liver of ABCG1 BAC Tg mice (N=2). (B) ABCG1 is overexpressed 6-fold in cortex, 6-fold in hippocampus and 3-fold in cerebellum of ABCG1 BAC Tg mice compared to wildtype (N=3). Western blots were summarized by densitometry and wildtype was defined as 1. (C) Human and murine ABCG1 expression is unaffected by either the presence of amyloid and the APP transgene in 13 month old PDAPP APP+ mice or the APP transgene alone in 3 month old APP+ mice when compared to either wildtype or ABCG1 BAC+ mice (N=2). * = p<0.05 Cortex (Cx), Hippocampus (Hp), Cerebellum (Cb)
influence ABC transporter expression\textsuperscript{158}. To determine if a similar confounding effect may be present after crossing the ABCG1 BAC Tg mice to the PDAPP AD mouse model of AD used in later experiments (2.2.5), brain levels of ABCG1 were also measured in ABCG1/PDAPP both prior to and after the onset of amyloid deposition. The Western blots indicate that robust overexpression of the ABCG1 transgene is retained in ABCG1/PDAPP mice in both hippocampus and cortex (Figure 2-7C). These results confirm that the ABCG1 BAC Tg mouse is suitable for testing the impact of ABCG1 overexpression on amyloid development in the PDAPP mouse model of AD.

2.2.4. Characterization of the ABCG1 BAC transgenic mice

To validate the functionality of the ABCG1 BAC in vivo and further study the role of ABCG1 in the brain, previously reported phenotypes of ABCG1 overexpression were assessed in the ABCG1 BAC Tg mice.

2.2.4.1. Cholesterol efflux to HDL is not increased from ABCG1 Tg astrocytes

The best established functional assay for ABCG1 function in cells is to facilitate cholesterol efflux specifically to lipid-rich apolipoproteins such as HDL and not to lipid-poor apolipoproteins. Importantly, although this function has been demonstrated in numerous studies using transfected cultured cells and ABCG1 knockout cells\textsuperscript{107,116,117} much less is known about the ability of physiological overexpression of ABCG1 to mediate efflux.

To determine if the ABCG1 BAC was functional in transgenic mice, we measured cholesterol efflux to HDL from primary astrocytes. Astrocytes were chosen for these experiments because they express Abcg1 and play an important role in maintaining lipidation of apolipoproteins in brain\textsuperscript{153,159,160}. Primary astrocytes were harvested from 1-2d post-natal mice and cells were grown in culture for 14 days prior to seeding for efflux experiments. In a typical cholesterol efflux experiment, cells of interest are loaded with excess cholesterol, which stimulates expression of genes including Abcg1 and Abca1 that are involved in promoting the efflux of this excess lipid. Cholesterol loading is thought to result in the production of
endogenous oxysterol ligands that activate the LXR/RXR pathway and stimulate expression of genes that restore cholesterol homeostasis. Therefore, astrocytes were cholesterol loaded with acetylated LDL (AcLDL) for 24 hours to stimulate Abcg1 expression prior to efflux. Cells were then washed, and cholesterol efflux was measured over 5 hours in either plain media, or in media containing apoAl as a positive control specific for ABCA1-mediated efflux, delipidated human apoE3, which is a natural cholesterol acceptor for ABCA1-mediated efflux from astrocytes and human HDL2/3 to detect ABCG1-mediated efflux. We expected that ABCG1 overexpression would result in increased cholesterol efflux to HDL2/3, but not to lipid-poor apoAl or apoE3.

Compared to baseline cholesterol efflux in media-only treated cells, we observed that cholesterol efflux to delipidated apoAl control was similarly increased by 1.75-fold (p=0.0006, N=6) in wildtype astrocytes and 1.8-fold (p=0.003, N=6) in ABCG1 BAC Tg astrocytes (Figure 2-8A). Cholesterol efflux to apoE was similar to that of apoAl. These results demonstrate that the ABCA1-specific pathway of cholesterol efflux is operative in primary astrocytes under these experimental conditions. Contrary to our expectations, the efflux of cholesterol from ABCG1 overexpressing astrocytes to HDL was non-significantly increased 1.2+/−0.2-fold (p=0.066, N=6) compared to wildtype astrocytes (Figure 2-8A). Because these data failed to show the expected increase efflux of cholesterol to HDL from ABCG1 overexpressing cells, we verified that our cholesterol loading protocol was up-regulating the expression of Abcg1 as intended.

2.2.4.2. Abcg1 expression is increased following acetylated LDL treatment

The AcLDL loading protocol used in these experiments was originally developed for macrophages to mimic exposure to oxidized-LDL that occurs in the artery wall, however astrocytes do not naturally accept lipids from oxidized LDL in vivo. LXR agonists such as TO901317 are also commonly used to induce ABCA1 and ABCG1 expression in a wide variety of cell types, including those typically used in cholesterol efflux assays. Therefore, primary astrocytes were incubated for 24 hours with either AcLDL in serum free media, regular
Figure 2-8: ABCG1 overexpression in primary astrocytes does not promote cholesterol efflux to HDL. (A) Efflux from either wildtype or ABCG1 BAC Tg primary astrocytes was measured for 4 hours in the presence of either plain media containing 0.2% BSA or supplemented with 15μg/mL delipidated ApoAl, 15μg/mL delipidated ApoE3, or 25μg/mL HDL. Data are pooled from 2 experiments done in triplicate (N=6) and are representative of 4 independent experiments. (B) ABCG1 QRT-PCR of primary astrocytes following treatment with basal media, basal media + 1μM TO901317 or 0.2%BSA +40μg/mL AcLDL. Human Abcg1 mRNA levels were determined in ABCG1 BAC+ mice and murine Abcg1 mRNA levels were measured in wildtype and ABCG1 BAC+ mice.
maintenance media that contains 10% FBS or maintenance media supplemented with TO901317. These conditions were chosen to represent efflux, baseline culture and induced conditions respectively. Following treatment human and murine Abcg1 mRNA levels were measured by QRT-PCR. Compared to no treatment, AcLDL incubation increased the expression of murine Abcg1 in wildtype astrocytes 5.7 ± 1.1-fold (p=0.013, N=3) and murine Abcg1 in ABCG1 BAC Tg astrocytes 12.2 ± 2.7-fold (p=0.016, N=3) and human Abcg1 in BAC Tg astrocytes 7.6 ± 0.7-fold (p=0.0009, N=3) (Figure 2-8B). LXR treated cells were observed to have similar levels of transcription induction to AcLDL treated cells (Figure 2-8B). These data confirm that AcLDL treatment increases Abcg1 expression in both wildtype and transgenic astrocytes in our efflux experiments and does not explain why Abcg1 overexpression did not increase cholesterol efflux to HDL.

2.2.4.3. Steady-state lipid levels are not changed in ABCG1 BAC Tg brain

An independently-generated ABCG1 BAC Tg model has been reported to have reduced levels of cholesterol and phospholipids in peripheral tissues when fed a high fat diet118. This finding was considered significant as it confirmed the established in vitro function of ABCG1 in vivo. We hypothesized that ABCG1 function in the brain and periphery would be similar, and because Abcg1 is highly expressed in brain, the lipid content of the brain may be reduced by ABCG1 overexpression. Because brain cholesterol levels are reported to be unaffected by dietary intake, chow-fed mice were analysed.

To determine if ABCG1 regulates the levels of cellular lipids in the brain as it is reported to in peripheral tissues, total brain lipid levels were measured. Lipid extracts were prepared from adult brains from ABCG1 Tg mice and littermate controls. Total lipid content was assessed following chloroform:methanol extraction and separation on a positive phase HPLC column. Lipid species were identified by retention time on the HPLC column and were quantified using a standard curve method. The levels of cholesterol esters, free cholesterol, ceramide, phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine,
phosphatidylcholine and sphingomyelin for ABCG1 BAC Tg and wildtype brains are summarized in Table 2-2. These results show that ABCG1 overexpression does not alter the abundance of these lipids in brain.

2.2.4.4. Cholesterol precursors and metabolites are reduced in ABCG1 Tg brains

The results describes in section 2.2.4.1 failed to detect ABCG1 facilitated efflux from primary astrocytes as expected. Total lipid levels measured in section 2.2.4.3 also failed to find any effect on total lipid levels in brain. However, overexpression of a human ABCG1 BAC had been reported to impact cholesterol homeostasis in peripheral tissues and expression of a murine BAC was reported to facilitate cholesterol efflux from murine macrophages, suggesting that physiological overexpression of ABCG1 facilitates lipid transport in vivo. The brain, however, is reported to be more resistant to disturbances in cholesterol homeostasis than the periphery, as it is insulated from the fluctuating intake of dietary cholesterol by the BBB and has been shown to tightly regulate cholesterol biosynthesis to maintain physiological levels despite pharmacological or genetic manipulations. Additionally, 70-80% of the cholesterol in the brain is present in myelin sheaths that is very stable and may obscure relatively small changes in membrane cholesterol levels in the pool of cholesterol that is metabolically active in the brain. To overcome the unique characteristics of cholesterol homeostasis in the brain, we performed cholesterol metabolite analysis that had previously been reported to detect small changes in cholesterol synthesis in the brain. Cholesterol metabolite analysis takes advantage of the fact that the brain synthesizes all of its cholesterol and disposes of excess cholesterol through enzymatic conversion by Cyp46A1 to the oxysterol 24S-hydroxycholesterol that passively diffuses across the BBB. Because of this closed-system physiology, a thorough analysis of cholesterol precursors and metabolites can detect small changes in cholesterol synthesis or elimination, in addition to measuring total cholesterol levels. Because Abcg1 expression is highest in murine embryos and juveniles and declines into adulthood and cholesterol synthesis and because cholesterol turnover in the
Total Brain Lipids

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Table 2-2: Total lipid levels in brain are not significantly affected by ABCG1 overexpression. Total lipid extracts were prepared from adult ABCG1 BAC transgenic and wildtype brains, quantified by HPLC and normalized to the protein content of the starting material. The presence of the ABCG1 BAC was not associated with any significant differences in lipid levels (p>0.05, N=4).

Mouse brain is estimated to peak at 14 days\(^{166,167}\), we hypothesized that cholesterol metabolism in the developing mouse brain would be highly sensitive to ABCG1 overexpression. To thus obtain the most sensitive measure possible, whole brains of 18 day and adult mice were subjected to cholesterol metabolite analysis. Whole brains were harvested, total lipid extracts were prepared by chloroform:methanol extraction and metabolite levels were determined by GC:MS. Compared to wildtype adult animals, cholesterol precursors in ABCG1
BAC transgenic brain were reduced. Notably, the precursor lathosterol was reduced by 21%(p=0.007, N=8), lanosterol was reduced by 14%(p=0.002 N=8) and desmosterol was reduced by 27% (p=0.009, N=8) and levels of cholesterol catabolite 24S-hydroxycholesterol was reduced by 15% (p=0.001, N=8) (Table 2-3). The plant-derived phytosterols Stigmasterol, Sitosterol, Sitostanol, Brassosterol, Campesterol and Campestanol and peripherally derived oxysterols, 7α-hydroxycholesterol, 27-hydroxycholesterol, were included in this experiment as controls for BBB integrity and plasma contamination. The levels of phytosterols and cholesterol catabolites in ABCG1 transgenic brain did not differ significantly from wildtype mice, confirming the validity of our observations (Table 2-3). Contrary to findings in the adult brain, no significant changes in cholesterol metabolite or sterols levels were observed between 18 day old wildtype and ABCG1 transgenic mice (Table 2-3). These results provide the first evidence that ABCG1 BAC is functional in vivo. Secondly these data suggest a previously unreported result that ABCG1 may modulate the levels of cholesterol precursors in vivo. That total cholesterol levels remain stable, despite a decrease in cholesterol precursors, may be explained by the decrease conversion of cholesterol to 24S-hydroxycholesterol. It was not clear from this experiment why ABCG1 influenced metabolite levels in the adult brain but not in very young brains.

2.2.4.5. Apolipoprotein E levels are unchanged in brain of ABCG1 transgenic mice

Numerous lines of evidence suggest that apoE in the brain is central to the progression of AD neuropathology. Furthermore, it has been reported that ABCG1 facilitates apoE secretion from cells and may influence total apoE levels. To determine if ABCG1 overexpression in vivo could impact apoE metabolism, we measured total and secreted apoE levels in brain of ABCG1 BAC mice. Whole brain levels of apoE in ABCG1 transgenic mice were unchanged compared to wildtype animals as determined by Western blot (Figure 2-9A). Secreted apoE in CSF was also measured by ELISA and was not significantly different between wildtype mice (1.34µg/mL ± 0.18) and ABCG1 BAC Tg mice (1.20µg/mL ± 0.12)
### Table 2-3: Cholesterol precursors and 24S-hydroxycholesterol levels are reduced in adult ABCG1 overexpressing brains but not very young mice.

Whole lipid extracts were prepared from brains of 100 day old chow-fed male mice or 18 day old male mice prior to wean. Sterol levels were determined by GC:MS and normalized to dry weight of starting material. (ns = p>0.05) N=8/genotype for all ages.
Figure 2-9: ApoE levels are not affected by ABCG1 overexpression. (A) Western blots prepared from whole brain lysates of ABCG1 BAC Tg mice and wildtype littermates were probed with antibodies specific ApoE (N=2). (B) ApoE levels in murine CSF collected from ABCG1 BAC Tg mice and wildtype littermates controls was determined by ELISA in triplicate (p>0.05, N=8). Both experiments are representative of 2 individual experiments.

(p=0.5, N=8) (Figure 2-9B). These results indicate that ABCG1 overexpression does not influence secreted or total levels of apoE in brain.

2.2.5. ABCG1 overexpression in vivo, Aβ production and amyloid

To determine whether ABCG1 overexpression could accelerate amyloid pathology in vivo, ABCG1 BAC Tg mice were crossed to the PDAPP mouse model of AD. PDAPP mice express human APP bearing the Indiana (V717F) FAD mutation in hippocampal and cortical neurons.
ARSproduction and amyloid formation were then measured in our experimental cohorts. Because newly generated Aβ is PBS-soluble and has a halflife of 2 hours in brain \(^{172}\), the PBS-soluble Aβ content of hippocampus was measured to estimate Aβ production. Amyloid is primarily composed of PBS-insoluble Aβ fibrils, so the Aβ content of guanidine soluble hippocampus extracts was used to estimate amyloid load. Although PBS extraction removes soluble Aβ species, the mass of fibrillar Aβ can be many orders of magnitude higher than the mass of soluble Aβ and can easily contaminate soluble Aβ measurements. To reduce the effects of insoluble amyloid on soluble Aβ measurements, mice were analyzed before amyloid deposition at 3 months of age and at 13 months of age, after amyloid deposits are reported in PDAPP brain.

2.2.5.1. ABCG1 overexpression does not influence amyloid deposition in vivo

Aβ species were serially extracted from hippocampi, first by PBS to remove soluble species, and secondly by guanidine hydrochloride to solubilize fibrillar amyloid into Aβ monomers. The levels of Aβ40 and 42 in PBS-soluble and guanidine-insoluble extracts were determined by ELISA and normalized to wet weight of starting material. At 3 months of age, PBS soluble Aβ42 levels in hippocampus of 3 month-old ABCG1 transgenic mice were increased 1.19-fold (p=0.039, N=18) compared to littermate controls (Figure 2-10B). However, PBS-soluble Aβ40 levels were not significantly affected by ABCG1 overexpression (p= 0.30, N=18) (Figure 2-10A). Furthermore, in 13 month old samples no significant differences were observed in PBS-soluble Aβ40 (p= 0.71, N=22) (Figure 2-10C) or Aβ42 (p=0.90, N=24) (Figure
Figure 2-10: Soluble and insoluble human Aβ levels in the hippocampus of PDAPP mice either wildtype or overexpressing ABCG1. (A, B) Hippocampal Aβ levels from 3 month old mice prior to amyloid formation (N=18). (C, D) Hippocampal Aβ levels in 13 month old animals with amyloid deposits (N=25). Samples were serially extracted first in PBS to measure soluble Aβ species followed by Guanidine hydrochloride (GuHCl) extraction to solubilize fibrillar amyloid. Aβ species were measured by human specific ELISAs. (A,C) Aβ40 and (B,D) Aβ42. All results are p>0.05 unless noted. * = p<0.05

Additionally, no significant changes in guanidine-soluble Aβ40 was observed at 3 months (p=.99, N=18) or 13 months (p= 0.71, N=22) (Figure 2-10A), nor did ABCG1 overexpression impact guanidine-soluble levels of Aβ42 at 3 months of age (p=0.21, N=18) (Figure 2-10B) or 13 months of age (p=0.87, N=23) (Figure 2-10D). These results show that 13 month old animals have ~50-fold more insoluble Aβ40 and 900-fold more insoluble Aβ42 than
3 month old animals, confirming the presence of amyloid at 13 months as expected. These data do not find a correlation between ABCG1 genotype and PBS-soluble or guanidine-soluble Aβ species with the exception of a 19% (p=0.039, N=18) increase of soluble Aβ42 in ABCG1 transgenic mice compared to controls at 3 months of age. Given the small magnitude of the change at 3 months and the failure to observe a corresponding increase in insoluble amyloid levels at 13 months, these data suggest that ABCG1 overexpression does not substantially impact amyloid burden in the PDAPP mouse model of AD at 13 months of age.

2.2.5.2. Total APP and C99 levels are not affected by ABCG1 overexpression

To determine if ABCG1 overexpression in vivo increased APP processing by β and α-secretase as observed in HEK:APP cells (Section 2.2.1.2, 2.2.1.3 and Figure 2-1, 2-2), C99 and C83 levels were measured in brain of ABCG1 BAC Tg mice. RIPA buffer tissue homogenates were prepared from 3 and 13 month old cortex and hippocampus of ABCG1 BAC Tg / PDAPP cohort described in section 2.2.5 and C83 and C99 levels were measured by Western blot. Because PDAPP mice are known to have variable levels of APP expression that could directly impact CTF levels, CTF levels were normalized to APP protein. In contrast to our in vitro results, APP and C99 levels in cortex and hippocampus were not significantly affected by ABCG1 over-expression at either 3 or 13 months of age (Figure 2-11A-D). Because the C83 band detected in these blots may be comigrating with other smaller CTF species (data not shown), an accurate determination of C83 levels cannot be made from this experiment. These results show that C99 production is not increased by the overexpression of ABCG1 in vivo. These findings largely confirm previous findings in section 2.2.5.1 that suggest Aβ production and amyloid formation are not substantially affected by the overexpression of ABCG1.

2.2.5.3. Murine Aβ levels are not affected by ABCG1 protein levels

To obtain an independent measure of Aβ production in brain, the levels of endogenous murine Aβ were also determined in ABCG1 BAC Tg mice in the absence of the PDAPP APP
Figure 2-11: C99 and total APP levels are not affected by ABCG1 overexpression. Tissue homogenates from hippocampus and cortex of PDAPP crossed ABCG1 BAC Tg mice were prepared by tris-tricine SDS-PAGE for Western blotting. Membranes were probed with antibodies specific to the C-terminus of human APP and the results were quantified by densitometry from duplicate gels for comparison. C99 levels are expressed as a ratio of total APP.
transgene. Murine APP is cleaved through similar mechanisms as human APP, however, because murine Aβ does not form amyloid deposits\textsuperscript{173,174} the levels of murine Aβ may more accurately reflect production of soluble Aβ species than the level of human Aβ observed in PDAPP mice. Furthermore, to thoroughly understand the role of ABGC1 in Aβ production, both ABCG1 deficient and ABCG1 Tg mice were evaluated in this experiment. To measure soluble murine Aβ levels, PBS homogenates of total brain from ABCG1 BAC Tg mice and wildtype littermates as well as ABCG1 +/-, +/- and -/- littermates were prepared and murine Aβ40 and 42 levels were determined by ELISA. Compared to wildtype littermates, ABCG1 BAC transgenice mice did not have significantly different levels of murine Aβ40 (p=0.25, N=6 wildtype, N=4 Tg) or murine Aβ42 p=0.34, N=6wildtype, N=4 Tg) (Figure 2-12A). Furthermore, compared to wildtype (+/+) littermates, we did not observe statistically significant changes in either murine Aβ40 levels in ABCG1 hemizygotes (+/-) (P<0.40, N=3) or ABCG1 deficient mice (-/-) (p<0.22, N=3) or changes in murine Aβ42 in hemizygotes (p=0.27, N=3) or ABCG1 deficient mice (p=0.18, N=3) (Figure 2-12B). These data suggest that neither ABCG1 overexpression, nor deficiency, has a significant impact on murine Aβ production in vivo.
Figure 2-12: ABCG1 overexpression or deficiency do not affect murine Aβ levels in brain. (A) Whole brains of ABCG1 BAC Tg mice and wildtype littermate controls (WT N= 6, Tg N=4) or were homogenized in PBS and the level of PBS soluble murine Aβ40 and Aβ42 were determined by ELISA. (B) Murine Aβ levels in brain of wildtype(+/+) , ABCG1 hemizygous(+-) and ABCG1 deficient(-/-) littermates (N=3). ns = p>0.05
3. DISCUSSION AND FUTURE DIRECTIONS

3.1. Introduction

The overall goal of this research was to test the hypothesis that ABCG1 plays an important role in the pathogenesis of Alzheimer's Disease. The research hypothesis is driven by the convergent observations that 1: Membrane cholesterol levels modulate Aβ production in neurons, a key event in amyloid formation. 2: The cholesterol transporter ABCG1 is highly expressed in brain and regulates cholesterol distribution and transport from cells suggesting that overexpression or deficiency of ABCG1 might impact the production of Aβ through a cholesterol-related mechanism. 3: Because ABCG1 overexpression occurs in DS, concomitant with accelerated amyloid deposition, we hypothesize that ABCG1 may be one of the genes on chromosome 21 capable of promoting amyloid pathology when overexpressed. Because of the presence of amyloid in individuals with DS, our experiments focused primarily on testing the hypothesis that ABCG1 overexpression would promote Aβ production from cells and promote amyloid formation in brain. The hypothesis was tested both in vitro using a cell-based assay and in vivo by crossing a murine model of AD with an ABCG1 overexpressing mouse developed in this thesis. Given that the ABCG1 mouse was a novel animal model the expression of the human ABCG1 transgene was characterized and lipid-related phenotypes were also assessed. Finally, the impact of ABCG1 deficiency on Aβ production was explored.

3.2. Discussion

3.2.1. ABCG1 overexpression and Aβ production in vitro

Using a cell-based assay, we showed that ABCG1 overexpressing cells produced more Aβ peptides of 40 and 42 amino acids in length, suggesting that an increase in β-secretase-mediated processing of APP may be the most likely underlying mechanism. The fact that only efflux-active ABCG1 was able to promote Aβ production suggests that the cholesterol transporting-activity of ABCG1 is essential for this process. Given a recent report that shows
ABCG1 overexpression increases the abundance of free cholesterol in the plasma membrane, this result is consistent with previous studies linking increased membrane cholesterol content with increased Aβ production in vitro. Because an increase in Aβ production can directly correlate to an increased amyloid burden in humans, our results are consistent with the hypothesis that ABCG1 overexpression accelerates amyloid formation. Unexpectedly, an increase in α-secretase activity was also observed. Because α and β secretase activity occur primarily at the cell surface, these phenomena are likely explained by the observation that ABCG1 overexpression increases the amount of APP present at the cell surface. These experiments demonstrate that ABCG1 overexpression in HEK cells increases the processing of APP by β-secretase into C99 and a corresponding increase in Aβ production.

These findings are in contrast with a recently published report that found ABCG1 overexpression reduced the secretion of Aβ in vitro. Both studies employ transient transfection assays however, the study by Kim and colleagues does not normalize secreted Aβ levels to control for cell number nor did the study measure CTF levels to verify increased APP processing. Given these shortcomings, Kim and authors cannot conclude that ABCG1 decreases Aβ production. Furthermore, Kim and colleagues used CHO-APP cells as opposed to HEK-APP cells that also may account for, but not explain, the observed differences in experimental outcomes.

One must use caution when interpreting these in vitro results however, as similar cell culture experiments analyzing the transporter ABCA1 reported that expression levels correlate with Aβ production, either positively or negatively depending on the study. Since the completion of the cell culture studies however, three further reports have been conducted that show ABCA1 deficiency in fact has no impact on Aβ production in vivo. These contradictory reports highlight the need to validate any results based on cell-models in vivo.
3.2.2. The ABCG1 BAC transgenic mouse

The ABCG1 overexpressing mouse carries a stably integrated BAC containing an insert of genomic DNA corresponding to the human Abcgl gene and flanking regulatory sequence. The use of a BAC is necessitated by the size of the 141 kb transgene that is too large to be contained in traditional bacterial vectors. The presence of cis-regulatory elements and preservation of the exon-intronic structure should ensure the natural protein isoforms are expressed in a physiologically regulated way, provided that the transcriptional machinery of the host organism is capable of recognizing and accurately binding the transgenic DNA. A very successful precedent for the use of genomic DNA is the generation of the ABCA1 transgenic mouse, which worked exceptionally well for the analysis of the effect of excess ABCA1 on HDL metabolism and atherosclerosis\textsuperscript{105,155}, as well as reports showing that several BAC transgenes were expressed in a coordinated way resembling the physiologically regulated gene, reviewed in\textsuperscript{154}. The caveats of the BAC approach are 1: high-degrees of overexpression can be difficult to obtain, as gene over-expression can lead to a compensatory down-regulation of the transgene and 2: The pattern of transgene expression will depend to some degree on host-specific expression of transcription factors. For instance, in the case of the ABCG1 BAC, the transgene is expected to be expressed in part through the binding of murine LXR/RXR and PPAR-\(\gamma\) transcription factors that may not have identical expression patterns in mice and humans\textsuperscript{108,112}.

Because the goal of these experiments was to determine the impact of increased Abcg1 gene dose on amyloid formation, we wanted to ensure the ABCG1 BAC was expressed in a physiological relevant way and that robust protein overexpression was achieved to facilitate detection of a phenotype. Physiological regulation of transgene expression was confirmed by 1: A survey of different tissues revealing that human and murine Abcg1 mRNA levels were similar in most tissues. 2: The observation that human and murine Abcg1 mRNA expression in primary neurons was induced to a similar degree by a synthetic LXR agonist and 3: Cholesterol loading of primary astrocytes induced the expression of both species of Abcg1 mRNAs to
similar degrees. Western blots that show ABCG1 protein is overexpressed 3-6-fold in peripheral tissues and brain regions of ABCG1 BAC Tg mice, confirming that human Abcg1 mRNA is translated into excess protein. In comparison, individuals with DS are reported to overexpress Abcg1 mRNA 1.5-2-fold and 2-fold at the protein level \(^{127,181}\). We also observed that the ABCG1 BAC remains robustly expressed in APP expressing mice with amyloid, which is an observation in contrast to ABCA1 BAC transgenic mice that subtly overexpress ABCA1 in brain in the absence of human APP, but lose this overexpression in the presence of amyloid \(^{158}\). Taken together these experiments confirmed that the human ABCG1 BAC transgenic mouse is a good model for studying the in vivo role of ABCG1.

3.2.3. Human and murine Abcg1 expression

A survey of murine Abcg1 expression was performed in 12 separate tissues. A comparison of the murine mRNA expression data with a previously published report of human Abcg1 mRNA abundance suggests that Abcg1 expression differs between mice and humans \(^{107}\). Specifically, the abundance of Abcg1 mRNA in human liver was similar to most tissues, whereas we observed that Abcg1 was poorly expressed in murine liver. This observation, however, may reflect differences between the average human diet and the chow fed mice in this study as cholesterol transporter expression is reported to be greatly increased in hepatic cells of mice fed a diet high in cholesterol and fat \(^{182}\). Additionally, in human brain, Abcg1 expression was reported to be lower than kidney, spleen, liver and lung, whereas we observed that murine brain had one of the highest levels of Abcg1 expression. These comparisons suggest that humans and mice may express Abcg1 at different relative levels in specific tissues.

3.2.4. Cell-specific expression of Abcg1 in brain

The knockout strategy employed to generate the Abcg1 knockout mice places a LacZ reporter construct under the control of Abcg1 regulatory sequence. Staining for the presence
of the reporter in Abcg1 +/- brain sections clearly indicated that Abcg1 was expressed in hippocampal layers primarily composed of neurons and cortical layers. Diffuse staining was also observed throughout the rest of the brain section shown. These findings are consistent with previous reports of Abcg1 expression in neuronal cell lines and embryonic neurons. In our experiments, LacZ staining appears to be most intense in hippocampal neurons, however, care must be taken to discriminate the intensity of staining from density of stained cells as densely packed cells may create the illusion of high expression. Astrocytes are reported to express Abcg1 and may represent some or all of the non-hippocampal staining however, no reliable comparison can be made between the level of neuronal and non-neuronal Abcg1 expression from these data.

3.2.5. The in vivo function of ABCG1

3.2.5.1. Cholesterol efflux:

The established function of ABCG1 is to facilitate cholesterol efflux to lipidated acceptors. To validate the functionality of the ABCG1 BAC in a cell type known to play an important role in cholesterol homeostasis of the brain, we measured the cholesterol efflux capacity of primary astrocytes. In these experiments ABCA1-specific controls confirmed ABCA1-mediated efflux, suggesting that the experimental conditions employed were sufficient to observe the export of cholesterol from cells. However, in contrast to previous reports that suggest ABCG1 is more important than ABCA1 for astrocyte cholesterol efflux, excess ABCG1 did not promote a statistically significant increase in cholesterol efflux to HDL. This result suggests three possible explanations.

Firstly, in primary cells expressing endogenous ABCG1, cholesterol efflux to HDL may not be a sensitive measure of ABCG1 function or ABCG1 may not be a rate-limiting step in cholesterol efflux. For example, complete knockout of ABCG1 protein in macrophages suggests that ABCG1 only accounts for 30-50% of the cholesterol efflux to HDL overall. Furthermore, very high levels of ABCG1 overexpression in immortalized cell types that lack
endogenous ABCG1 typically produce a 30-40% increase in cholesterol efflux to HDL suggesting that the relatively modest 3-5-fold overexpression of ABCG1 obtained in this experiment may not be easily detectable, especially in the context of endogenous ABCG1 expression.

The second explanation for the absence of efflux activity in primary astrocytes may reflect the in vivo function of ABCG1. The vast majority of overexpression studies, including the HEK studies described here, use a human cDNA corresponding to an mRNA isoform containing exons 5, 7, 11-23. This isoform is highly induced by oxysterol treatment, however, not all Abcg1 isoforms have been conclusively shown to promote cholesterol efflux. As the Abcg1 isoforms expressed in brain have not been described here or elsewhere, it is possible that the Abcg1 isoforms expressed from the physiologically regulated ABCG1 BAC are either less effective or ineffective at promoting cholesterol efflux from astrocytes. It is unlikely that the lack of ABCG1 mediated efflux in these experiments is entirely related to the use of astrocytes as the ABCG1 BAC also fails to promote cholesterol efflux from primary macrophages that are known to utilize ABCG1 mediated pathways for cholesterol efflux (Burgess BL., et al. manuscript submitted to J. Lipid Res; Edwards PA., Principal Investigator, personal communication).

The third explanation for the failure to observe ABCG1 facilitated efflux from either ABCG1 overexpressing astrocytes or macrophages (Burgess BL., et al. manuscript submitted to J. Lipid Res) may represent differences or incompatibilities between murine and human ABCG1. This is supported by the observation that macrophages harvested from BAC Tg mice generated with murine Abcg1 have elevated cholesterol efflux to HDL. However two groups, including ours, that generated BAC Tg mice with human Abcg1 failed to observe an increase of cholesterol efflux to HDL from macrophages or astrocytes. This suggests that human ABCG1 may not function properly in mice (Burgess BL., et al. manuscript submitted to J. Lipid Res; Edwards PA., Principal Investigator, personal communication). Although murine and human ABCG1 are 97-98% identical at the protein level depending on the particular
protein isoform, the 2-3% difference accounts for ~10-25 non-identical amino acids between the species. The incompatibility could be a result of improper dimerization of murine and human ABCG1 or improper trafficking of human ABCG1 in murine cells. For example, single missense mutations in ABCG5 or ABCG8 are reported to prevent the assembly of functional G5/G8 heterodimers and impair proper sub-cellular trafficking\textsuperscript{184}, suggesting that small differences between human and murine ABCG1 may prevent, or impair the assembly of functional human/murine ABCG1 dimers. Although human Abcg1 cDNAs have been shown to facilitate efflux in murine cell types, these experiments are typically conducted under conditions where human ABCG1 is greatly overexpressed and would be largely present in human/human homodimers with human/murine dimers forming a minor component. As we do observe a gain of function in ABCG1 overexpressing mice with respect to the levels of cholesterol precursors, that activity may be preserved in murine/human ABCG1 dimers, or reflect the contribution of human/human dimers.

3.2.5.2. Brain lipid levels

As the ABCG1 protein is reported to protect against diet-induced accumulation of cholesterol and phospholipids in lung and liver, we investigated the lipid content of ABCG1 overexpressing brain\textsuperscript{118}. Our experiments did not involve any dietary manipulation and the BBB would prevent dietary lipids from accumulating in the brain. However, we hypothesized that because murine Abcg1 expression in brain is 2-6-fold higher than in lung and liver respectively, the steady-state level of lipids in ABCG1 overexpressing brain may be reduced. Despite the high level of expression in brain, the total levels of cholesterol ester, free cholesterol, free-fatty acids, sphingomyelin, ceramide and all phospholipids species examined were not changed, despite reports that ABCG1 can facilitate efflux of cholesterol, phosphatidylcholine and sphingomyelin from cells\textsuperscript{107,185}. The possibility remains that the cellular distribution within the brain of one or more of these lipids is affected.
3.2.5.3. Cholesterol metabolites in brain of ABCG1 BAC transgenic mice

We observed a reduction in cholesterol precursors and 24S-hydroxycholesterol in ABCG1 overexpressing adult mice, however, total cholesterol levels were not significantly changed. Specifically, a reduction in lanosterol, desmosterol and lathosterol precursors and 24S-hydroxycholesterol was observed. The cholesterol biosynthetic pathway is reviewed in Figure 1-2. Intriguingly and consistent with this finding, Abcg1 -/- mice are reported to accumulate desmosterol and lathosterol in brain (Wang N., Associate Research Scientist, personal communication). One interpretation of these results is that the activity of ABCG1 suppresses cholesterol biosynthesis through a mechanism related to the intracellular distribution of cholesterol. HMGR transcription is regulated in part by the sterol-sensing protein SCAP in the ER \cite{186}, suggesting that ABCG1 could regulate cholesterol biosynthesis by controlling the cholesterol content of the ER membrane. Alternatively, as small quantities of non-hydroxylated cholesterol are capable of being cleared across the BBB into the peripheral circulation through an unknown mechanism \cite{50}, ABCG1 may promote the clearance of one or more cholesterol precursors from the brain. In both of these models cholesterol levels in the brain could remain unchanged through a compensatory decrease in enzymatic conversion of cholesterol into 24S-hydroxycholesterol for elimination, which is supported by the data.

The failure to observe changes in cholesterol precursor or metabolite levels in very young mice may provide additional insights into the underlying mechanism. In very young mice cholesterol synthesis is occurring rapidly, and although the relative contributions of glia and neurons are not well understood, it has been suggested that astrocytes may play a smaller role in cholesterol synthesis in the developing brain than in the adult brain \cite{187,188}. Other studies have reported that the majority of cholesterol is synthesized through the desmosterol pathway in the developing brain whereas the lathosterol branch is primarily used in the adult brain \cite{189}. These studies suggest that age-related differences in cholesterol metabolism or age related differences in cell-specific expression patterns of Abcg1 may explain this discrepancy.
3.2.6. ABCG1 and ApoE

The level of total-brain apoE and secreted apoE in CSF were not significantly changed by overexpression of the ABCG1 BAC. These findings fail to support one study that reported siRNA inhibition of ABCG1 reduced apoE secretion from human monocyte derived macrophages. It is not clear from our experiments whether the discrepancy reflects differences between macrophages and glia, or the effect of overexpression versus knockdown.

3.2.7. ABCG1 and AD

3.2.7.1. ABCG1 and amyloid

To test whether ABCG1 overexpression could influence the production of Aβ and produce a corresponding increase in amyloid formation, the ABCG1 overexpressing mice were crossed to the PDAPP mouse model of AD that express human APP and develop amyloid deposits. We measured PBS-soluble Aβ to estimate newly synthesized Aβ and the PBS-insoluble-guanidine-soluble pool to measure Aβ deposited as amyloid fibrils. We expected to observe an increase in Aβ production in ABCG1 overexpressing mice and a corresponding increase in amyloid in older mice consistent with our hypothesis and our in vitro findings. However, our results show that ABCG1 overexpression was only associated with a 19% increase in soluble Aβ42 levels and this was specifically observed in amyloid-free hippocampus. No significant effects were seen on Aβ40. Furthermore, despite the increase in soluble Aβ42 at three months of age, a corresponding increase in amyloid Aβ42 was not observed in older animals, suggesting that the small increase is not sufficient to affect murine amyloid formation over this timeframe. In our HEK experiments, we observed that the increase in Aβ production was coupled with an increase of APP processing into C99 by β-secretase. However, in vivo, ABCG1 overexpression did significantly impact C99 levels in cortex or hippocampus. This would suggest that subtle changes in C99 levels were below our sensitivity threshold, or that the increase in Aβ42 in young mice may occur through an alternate pathway, possibly involving γ-secretase. Because we observed no change in CTF levels, these results
suggest that overexpression of ABCG1 in the brain is not sufficient to increase Aβ production, indicating that much more ABCG1 may be required to affect Aβ production as in the experiments involving HEK-APP cells. It should be noted that difficulties in detecting changes in steady state Aβ levels could also be explained by increased Aβ clearance matching increased Aβ production, resulting in no net change. Taken together, ABCG1 overexpression may slightly increase Aβ42 production through a non-β-secretase mediated pathway, however these effects are not sufficient to impact amyloid levels in vivo.

3.2.7.2. ABCG1 and Murine Aβ

Murine Aβ levels were measured in the absence of human APP as an alternative measure of Aβ production. As murine APP lacks FAD mutations, does not form amyloid, and is endogenously regulated in contrast to PDAPP mice that express APP under the PDGF-β promoter, murine Aβ levels may provide a more accurate measure of Aβ production. A trend towards lower murine Aβ levels was observed in ABCG1 deficient mice that may achieve statistical significance in an experiment comprised of more than 3 animals per group. The relevance of this effect on amyloid formation cannot be determined by this experiment as murine Aβ does not accumulate as amyloid over the lifespan of the mouse. Consistent with the overall findings from the previous experiment in PDAPP mice, ABCG1 overexpression failed to make a significant impact on murine Aβ levels. These experiments further suggest that ABCG1 does not significantly impact Aβ production.

3.2.8. Conclusion: The function of ABCG1 in brain

This research described the generation of a novel ABCG1 overexpressing murine research model. The ABCG1 BAC transgenic mouse possesses human genomic Abcgl under the control of endogenous cis-regulatory sequence and preserves intronic/exonic structure. The transgene was expressed in a similar tissue specific pattern as murine Abcg1, which we have shown may differ from Abcg1 expression in humans. Additionally, this research provides
the first evidence that Abcg1 is well expressed in adult hippocampal neurons and confirms ABCG1 expression in cultured embryonic neurons and post-natal astrocytes. This research produced novel data suggesting that ABCG1 may modulate cholesterol precursor and metabolite levels in brain, although the underlying mechanism has yet to be fully determined. Furthermore, ABCG1 overexpression did not facilitate cholesterol efflux from astrocytes, suggesting that physiological overexpression of ABCG1 may have different consequences on cholesterol metabolism compared to high-level constitutive overexpression in cell-lines. ABCG1 overexpression also did not influence steady state levels of lipids in brain. In conclusion, this thesis describes the generation of a novel transgenic mouse overexpressing human ABCG1 and suggests a novel role for ABCG1 in cholesterol precursor metabolism in the brain.

3.2.9. Conclusion: The role of ABCG1 in AD

This research was based upon the proposed ability of ABCG1 to influence cellular cholesterol homeostasis in neurons and the correlation between neuronal cholesterol levels and Aβ production. Because we observed reduced 24S-hydroxycholesterol levels in ABCG1 overexpressing brain and the production of 24S-hydroxycholesterol is reported to occur specifically in neurons, it can be inferred that ABCG1 overexpression influences cholesterol metabolism to some extent in neurons. However, the observations that murine Aβ levels are not affected by ABCG1 overexpression or deficiency, and that ABCG1 overexpression does not affect amyloid deposition or β-secretase cleavage of APP, provides compelling evidence that ABCG1 protein levels do not substantially affect Aβ production or amyloid pathology. This research concludes that ABCG1 is unlikely to participate in the rapid onset of AD pathology in DS.
3.3. Future Directions

The conclusion of this thesis is that ABCG1 overexpression is unlikely to play an important role in amyloid formation in AD, however, its potential influence on cholesterol metabolism remains important to AD, brain injury and other forms of neurodegeneration, warranting further study of the role of ABCG1 and other cholesterol related genes in the brain.

3.3.1. Determine how ABCG1 regulates cholesterol metabolite levels

The underlying mechanism through which ABCG1 overexpression reduces cholesterol precursors and 24S-hydroxycholesterol was not determined in this thesis. As cholesterol synthesis is primarily regulated through the abundance of HMGR protein, Western blots of brain tissue would determine whether ABCG1 modulates HMGR activity and is an appropriate next step for this line of investigation. Western blots from both mice and individuals with DS samples could be easily analyzed to confirm and extend the findings to humans.

3.3.2. Determine the cell-specific patterns of expression in brain

The primary cell types that express Abcg1 in the adult brain were not rigorously explored in this thesis. Immunofluorescence studies that co-label neurons, astrocytes or microglia in addition to ABCG1 would allow the cell-specific patterns of ABCG1 expression to be determined, however this was impossible prior to obtaining the ABCG1 knockout mice from Deltagen as all commercially available antibodies to ABCG1 perform poorly in immunofluorescence applications. In this thesis the LacZ reporter gene was detected by utilizing the catalytic activity of the β-galactosidase enzyme to convert X-gal to a colored precipitate, however, immunological detection of the LacZ reporter gene is also possible. By co-staining with monoclonal antibodies to the LacZ reporter gene and cell-type specific antibodies the regional and cell-specific patterns of ABCG1 expression could be determined in detail.
3.3.3. Determine the relevant \textit{Abcg1} isoforms in brain and their function

This thesis raised questions about the in vivo function of ABCG1 in the brain. As not all ABCG1 protein isoforms have been shown to induce cholesterol efflux and this study failed to observe ABCG1 mediated efflux from astrocytes, this discrepancy may be explained by determining the major \textit{Abcg1} isoforms in brain. It is likely that neurons and glia express different isoforms and the relevant isoforms may vary throughout development or in response to oxysterols and PPAR-\(\gamma\) ligands. The transgenic ABCG1 model developed in this study offers the unique potential to study ABCG1 mRNA isoform expression during development and in response to treatment by various agonists, using both in vivo and primary cell culture approaches. Once cloned, these ABCG1 mRNA isoforms, which are predicted to differ primarily at the N-terminus, could be screened for an ability to promote efflux or redistribution of cholesterol and other lipids reported to be transported by ABCG1 such as sphingomyelin and phosphatidylcholine. It is possible that alternative splicing could confer alternative substrate specificity, or could affect intracellular-trafficking of ABCG1.

3.3.4. Test the functionality of the human ABCG1 BAC in ABCG1 knockout mice

The hypothesis suggested in section 3.2.5.1 that human ABCG1 may be functionally impaired in mice as a result of improper dimerization with murine ABCG1, or incorrect intracellular trafficking could be tested. One approach to testing the hypothesis involves backcrossing the ABCG1 BAC onto the ABCG1-/- mice to determine if the ABCG1 BAC could rescue the impaired efflux-phenotype reported in ABCG1 knockout mice. A comparison of ABCG1 BAC hemizygotes and homozygotes in conjunction with \textit{Abcg1} -/-, -/+ and +/+ genotypes would determine whether human ABCG1 BAC is functional in mice, both in the presence and absence of murine ABCG1.
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