THE ROLE OF THE ATP-BINDING CASSETTE TRANSPORTER A1 IN ALZHEIMER DISEASE NEUROPATHOLOGY AND BRAIN LIPOPROTEIN METABOLISM

by

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ABSTRACT

The ATP-Binding Cassette transporter A1 (ABCA1) is a ubiquitously expressed protein that mediates the efflux of cholesterol and phospholipids from the plasma membrane onto lipid poor apolipoproteins (apos), such as apoA-I and apoE. Outside the central nervous system (CNS), this process constitutes the rate-limiting step in the generation of high-density lipoproteins (HDL). Low levels of HDL and cellular lipid accumulation are the hallmarks of Tangier disease, a genetic disease caused by mutations in the *ABCA1* gene. ABCA1 is also expressed in the brain, the most cholesterol rich organ in the body. However, previous to this thesis, the role of ABCA1 in brain cholesterol metabolism had been poorly explored. Furthermore, recent data indicated that disturbances in cholesterol homeostasis in the CNS may play an important pathogenic role in the development of Alzheimer Disease (AD). This, in turn, suggested that ABCA1 may also affect the progression of AD. The overall goal of this work is to gain insights into the role of ABCA1 in the development of AD neuropathology and brain lipoprotein metabolism.

This thesis presents original data showing that ABCA1 deficiency results in a dramatic reduction in brain apoE levels. Because apoE has a demonstrated role in amyloid deposition, we also studied the effects of ABCA1 deficiency on amyloidogenesis in AD transgenic mice. We found that the absence of ABCA1 results in increased amyloid deposition despite low levels of apoE. The observation that ABCA1-deficiency is proamyloidogenic raised the question whether ABCA1 overexpression may reduce amyloid formation. We thus evaluated AD neuropathology in AD mice crossed to an *ABCA1* bacterial artificial chromosome (BAC) transgenic mouse model. We found that although ABCA1 expression and apoE levels are elevated in the brains of *ABCA1* BAC mice compared to non-transgenic controls, these effects were abolished in the presence of AD transgenes. This suggests that overexpression of

physiologically regulated ABCA1 may be altered in brains with amyloid deposits compared to amyloid-free brains. These studies thus constitute original contributions to our understanding of the role of ABCA1 in brain lipid metabolism, and highlight the mechanisms by which ABCA1 may impact the development of AD neuropathology *in vivo.*

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LIST OF ABBREVIATIONS

ABCA1: ATP-binding cassette transporter A1

Aβ: amyloid β

AD: Alzheimer Disease

ApoA-I: apolipoprotein A-I

ApoE: apolipoprotein E

ApoER2: apolipoprotein E receptor 2

ApoJ: apolipoprotein J

ApoD: apolipoprotein D

APP: β amyloid precursor protein

CAA: cerebral amyloid angiopathy

CNS: Central Nervous System

CSF: cerebrospinal fluid

CTF: C-terminal fragments

FAD: familial Alzheimer Disease

FHA: familial hypoalphalipoproteinemia

HDL: high-density lipoprotein

LDL: low-density lipoprotein

LDLR: low-density lipoprotein receptor

LOAD: late-onset Alzheimer Disease

LRP: LDLR related protein

LXR: liver X receptor

NBD: nucleotide binding domain

NFT: neurofibrillary tangles

PS: presenilin

RXR: retinoid X receptor

s-APPα: secreted APPα fragment

s-APPβ: secreted APPβ fragment

TD: Tangier Disease

VLDL: very low- density lipoprotein

VLDLR: very low-density lipoprotein receptor

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CO-AUTHORSHIP STATEMENT

All studies described in this thesis were designed and performed by the author with the following exceptions:

Murine Aβ40 levels in Figure 3-3 were measured by Dr Jean-Francois Blaine. All studies reported in Section 5.1 were done by Karlygash Abildayeva and colleagues, except for figure 5.1 carried out by the author. Similarly, all studies described in Section 5.2 were carried out by Tansley, Burguess and colleagues, with the noted exception of figure 5.2, performed by the author.

Chapter 1 – Introduction

1.1 Overview

The overall goal of this thesis is to address the role of the cholesterol transporter ABCA1 in the development of Alzheimer Disease (AD) neuropathology. AD is a cortical neurodegenerative disease and the leading cause of dementia in developed countries [1]. Several observations indicate that genes involved in cholesterol metabolism, such as the cholesterol transporter ABCA1, may play a pivotal role in the development of AD. The brain is the most cholesterol-rich organ in the body [2], and maintenance of neuronal cholesterol homeostasis is critical for synaptic remodeling and recovery after acute and chronic damage [3]. In addition, cellular cholesterol levels modulate the generation of Aβ peptides, the toxic species that accumulate in the brains of AD patients [4]. The major cholesterol carrier in the brain is apolipoprotein E (apoE) [5], a known acceptor for ABCA1-mediated lipid efflux [6], and genetic variations of apoE represent the only well-validated risk factor for sporadic AD [7, 8]. ABCA1 is highly expressed in the brain [9], and transcriptional agonists of ABCA1 have been shown to increase cholesterol efflux in CNS-derived cells [10, 11]. Of interest to this thesis, synthetic transcriptional agonists of ABCA1 and other genes involved in lipid metabolism have been shown to decrease Aβ generation in murine models of AD [12, 13]. However, the specific role of ABCA1 in AD development and brain lipid metabolism was virtually unexplored prior to 2004. The work described in this thesis represents my contribution to this field and is presented herein as three primary research papers, all of which have been published.

The primary biochemical function of ABCA1 is to efflux excess cellular cholesterol onto apolipoprotein A-I (apoA-I), as the first step in HDL biosynthesis [6]. In humans and mice, ABCA1-deficiency results in very low levels of circulating HDL and apoA-I, and the little remaining plasma apoA-I is poorly lipidated and rapidly catabolized [14]. Although brain

expression of ABCA1 had been previously reported [9, 15], the exact role of this transporter in CNS lipoprotein metabolism was unknown. ApoE is the main apolipoprotein in the brain and is capable of inducing ABCA1-mediated lipid efflux. However, the exact relationship between ABCA1 and apoE in the brain was poorly understood. It was therefore of interest to evaluate whether the absence of ABCA1 would affect lipidation and levels of CNS apoE (paralleling its effects on apoA-I in the periphery). My thesis work demonstrates that the absence of ABCA1 indeed results in decreased cholesterol efflux of CNS-derived glia and accumulation of cellular lipids. Furthermore, brain apoE levels are dramatically reduced, mimicking the effects of ABCA1 absence on peripheral apoA-I.

ApoE has been directly implicated in the development of AD neuropathology as a chaperone that modulates Aβ deposition and amyloidogenesis. ApoE-deficient mice are unable to develop one of the hallmarks of AD, known as amyloid plaques [16]. This observation demonstrates that apoE is required to form the amyloid core of these plaques – at least in mice. Furthermore, amyloid burden is influenced by apoE levels, with apoE heterozygous mice displaying an amyloid load midway between those of apoE-deficient and wild-type animals [17]. Our first study demonstrated that ABCA1-deficiency dramatically reduces brain apoE levels, potentially reducing amyloid formation. Our next objective, therefore, consisted of the next logical step that was to evaluate the effect of ABCA1-deficiency on amyloid formation in murine models of AD. To our surprise, we found that the absence of ABCA1, with its consequent reduction in apoE levels, resulted in at least as much amyloid formation as in the presence of ABCA1 and wild-type levels of apoE. Although the lipidation status of apoE had previously been shown to affect Aβ binding [18, 19], our observation that ABCA1-mediated lipidation of apoE affects amyloid levels was the first *in vivo* demonstration that apoE lipidation plays a key role in amyloidogenesis.

Because the lack of ABCA1 enhanced amyloid load, my final study was to determine whether an increased gene dose of ABCA1, expressed under physiological regulatory elements, would

increase apoE levels or lipidation and thereby reduce amyloid levels *in vivo*. For this, *ABCA1* bacterial artificial chromosome (BAC) transgenic mice were crossed to a murine model of AD. We observed that *ABCA1* BAC transgene expression results in a very modest increase in both ABCA1 and apoE protein levels in the absence of the APP and PS1 genes. However, when crossed to the APP/PS1 mouse model of AD, expression of the *ABCA1* BAC transgene did not affect AD neuropathology, apoE or ABCA1 abundance. Additional observations in these mice provide intriguing preliminary data suggesting that posttranscriptional mechanisms may inhibit ABCA1 protein overexpression in the presence of AD neuropathology. The results of this third study clearly demonstrate that physiological expression of human *ABCA1* does not prevent amyloid deposition *in vivo*, and suggests that non-physiological methods to drive high levels of ABCA1 expression, such as heterologous promoter systems, may be required to reveal any potential protective effect of ABCA1 on AD neuropathogenesis.

In summary, the main hypothesis driving my thesis work is that **ABCA1 plays a key role in the development of AD neuropathology and in brain lipid metabolism.** This question was addressed in three specific aims:

- <u>Aim 1:</u> To evaluate the impact of ABCA1 deficiency on brain apoE and cholesterol metabolism.
- <u>Aim 2:</u> To assess the effect of ABCA1 deficiency on the development of AD neuropathology.
- <u>Aim 3:</u> To determine the influence of ABCA1 overexpression on both brain apoE metabolism and AD neuropathology.

1.2 Alzheimer Disease

1.2.1 Definition

On November 4, 1906, Alois Alzheimer described for the first time the clinical and neuropathological characteristics of the mental disorder that today is recognized as the most common cause of dementia in humans. Clinically, Alzheimer described several signs of advanced mental failure, such as reduced comprehension and memory, aphasia, disorientation, unpredictable behavior, paranoia, auditory hallucinations, and pronounced psychosocial impairment [20]. At the histological level, the German psychiatrist recognized the presence of abnormally prominent and thick neurofibrils, extensive neuronal loss and milliary foci of a 'peculiar substance' throughout the cortex [21, 22]. Today, one hundred years later, Alzheimer's initial clinical and neuropathological observations still constitute the cornerstone for the diagnosis of Alzheimer Disease (AD).

According to the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA), the definitive diagnosis of AD must meet both clinical criteria for AD and histological confirmation [23]. The clinical diagnosis of AD is based on the presence of a dementia syndrome (defined as a progressive decline in at least two cognitive domains including memory) with an onset between 40 and 90 years of age, in the absence of other medical or neurological explanations. The neuropathological confirmation of AD requires the presence of both neurofibrillary changes and amyloid deposits, which progress in a characteristic pattern. AD lesions are evident earliest in the entorhinal cortex, then spread through the hippocampal formation and isocortex, and then extend into the neocortex [24].

Neurofibrillary changes are detected by silver stains and result from the aggregation of hyperphosphorylated cytoskeletal proteins [24-26]. Amyloid deposits, on the other hand, are composed of amyloid β peptides (Aβ) [27] and Aβ deposits are classified into diffuse and neuritic plaques. Diffuse plaques are composed of non-fibrillar Aβ deposits and do not exhibit dystrophic neurites, whereas neuritic plaques are usually composed of a compact, fibrillar amyloid core surrounded by dystrophic neurites.

Aβ□ peptides derive from sequential proteolytic cleavage of the amyloid precursor protein (APP) (reviewed in [25]) (Figure 1-1). Human APP is ubiquitously expressed and has three major isoforms of 695, 751, and 770 amino acid residues. APP695 is the main neuronal isoform. APP corresponds to a type I transmembrane protein with its N-terminal localized in either the lumen of secretory vesicles or the extracellular space and its C-terminal facing the cytosol. Mature APP is processed by either one of two competing pathways: a non-amyloidogenic pathway that precludes the formation of Aβ and an amyloidogenic pathway that generates Aβ (Figure 1-1). Approximately 90% of wild-type APP is processed by the non-amyloidogenic pathway, in which APP is first cleaved by an α-secretase at a site located 12 amino acids N-terminal of the transmembrane domain. This cleavage generates a large, soluble, secreted ectodomain (APPs-α) and a membrane-bound C-terminal fragment (CTF) of 83 residues (C83). In the alternative amyloidogenic pathway, APP molecules are cleaved by β-secretase at 18 amino acids N-terminal of the α cleavage site, generating a secreted APPs-β domain and a CTF of 99 residues. α and β cleavages are mutually exclusive and are each followed by a γ-secretase cleavage within the transmembrane domain of APP. This process then originates either a p3 fragment (derived from α and γ cleavage) or an Aβ peptide ranging from 37-43 amino acids in length (when β-secretase proteolysis is followed by the action of γ - secretase). Of these Aβ peptides, Aβ40 and Aβ42 are quantitatively the most important. Compared to Aβ40, Aβ42 is more hydrophobic, aggregates more easily and is thought to constitute most of the immature

plaques found in AD. These immature plaques subsequently progress to neuritic plaques by recruiting Aβ40 [28]. A recent study demonstrated that Aβ42 is required for the formation of amyloid, further strengthening the hypothesis that Aβ42 deposits act as a nidus for subsequent amyloid formation [29]. Many genetic mutations that result in familial early-onset AD (FAD) increase the proportion of APP that is cleaved along the amyloidogenic pathway, or increase the ratio of Aβ42/Aβ40 that is produced along this pathway [30].

The enzyme responsible for the β-secretase activity was identified in 1999 as a novel membrane-bound aspartic protease and named β-site APP-Cleaving Enzyme (BACE) [31-34]. As for α-secretase activity, three members of a family of glycoproteins containing A Disintegrin And Metalloprotease domain (ADAM), namely ADAM-9, -10 and -17, have been implicated in both constitutive and inducible α-cleavage [35]. Finally, a multiprotein, high-molecular-weight complex has been demonstrated to be responsible for γ-secretase activity (reviewed in [36]). Presenilin-1 or 2 (PS), Nicastrin, APH-1 and Presenilin-Enhancer (PEN-2) have been identified as the four key components of the γ-secretase complex. Notably, APP and all of the individual components of each secretase are integral membrane proteins. Furthermore, the proteolytic activity of γ-secretase takes place within the hydrophobic membrane domain and is modulated by cellular cholesterol levels [4]. These observations suggest that alterations in the neuronal lipid environment may have considerable consequences for APP processing.

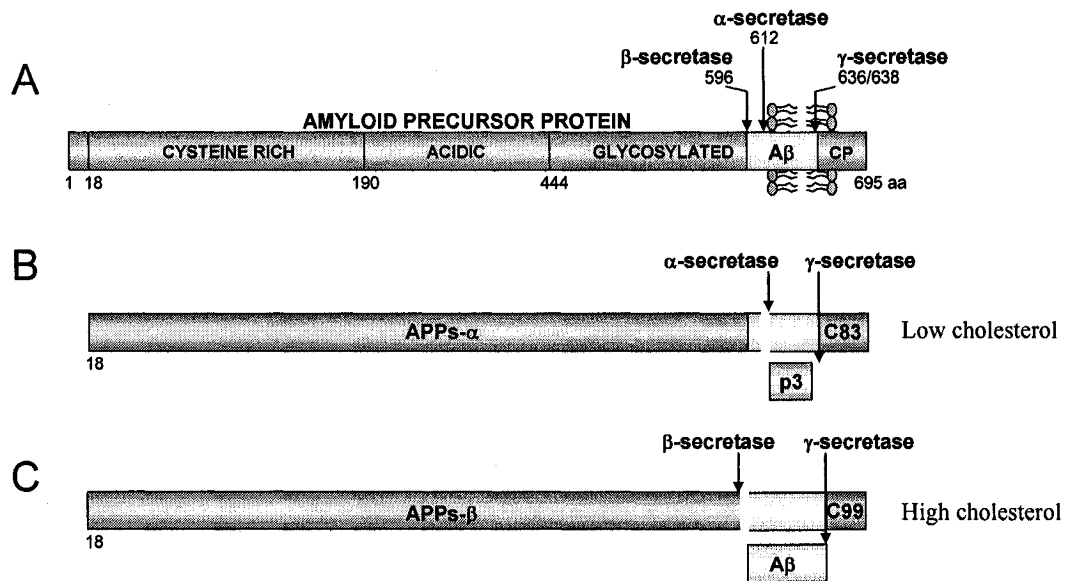

Figure 1-1. Schematic diagrams of amyloid precursor protein (APP) and its major metabolic derivatives.

(A) APP contains a 17 aa signal sequence, which is removed during transit through the secretory pathway. The remainder of the APP protein contains a cysteine-rich domain, an acidic domain, a glycosylated domain, the Aβ domain, and a cytoplasmic (CP) domain. The location of the transmembrane domain of APP is indicated by the phospholipid bilayer. APP is cleaved by three major secretases, including β-secretase, α-secretase, and γ-secretase. (B) Cleavage of APP by α-secretase releases a large soluble ectodomain of APP (αAPPs) and retention of an 83-aa C-terminal fragment (C83) in the membrane. Further cleavage of the C83 fragment by γ-secretase releases the p3 peptide. This pathway is favored under conditions of low intracellular cholesterol. (C) Cleavage of APP by β-secretase generates a truncated version of soluble APP (βAPPs) and a 99-aa C-terminal fragment. Further cleavage of the C99 fragment by γ-secretase releases Aβ peptides, the majority of which are 40 or 42 aa in length. This pathway is favored under conditions of high intracellular cholesterol.

1.2.2 The amyloid cascade hypothesis of Alzheimer Disease

Historically, of all the hypotheses that have been put forward to account for the cause of AD, the two most favored and controversial ones were the amyloid cascade hypothesis and the tau and tangle hypothesis. 'Tauists' sustained that the key insult in AD corresponds to the formation of neurofibrillary tangles (NFT). The observations that NFTs occur in neurons, apparently result in neuronal death, correlate well with the clinical progression of AD, and that NFT formation occurs in areas known to be critical for memory, supported this hypothesis [37]. 'Baptists', on the other hand, argued that the accumulation of Aβ was the causative factor of

AD. Aβ peptides are neurotoxic in a variety of experimental paradigms (reviewed in [25]) and mutations in the APP and PS genes, which result in increased generation of Aβ peptides, cause familial forms of AD (reviewed in [30]). Furthermore, Down's Syndrome patients, who inevitably present with AD neuropathology by age 40, inherit an extra copy of chromosome 21 on which the human *APP* gene is located [24]. The controversy between both hypotheses was lessened by the discovery of mutations in tau that cause specific types of fronto–temporal dementia (FTD), where neuronal tangles and neurodegeneration develop in the absence of amyloid plaques [38]. In contrast, mutations in APP and/or PS give rise to both plaques and tangles. Together, these observations argue very strongly in favor of altered Aβ metabolism occurring upstream of NFT development and as a key pathogenic factor in AD.

Because one of the main objectives of my thesis work was to resolve the experimental questions in a biologically relevant paradigm, amyloid-based murine models of AD were selected for these studies. They constitute a relevant *in vivo* model to test the proposed hypotheses, and, although imperfect, they recapitulate more AD-specific neuropathological features than tau-based ones [39]. In addition, and as described in detail below, experimental evidence suggests that Aβ metabolism may be sensitive to alterations in CNS lipid homeostasis[4].

1.2.3 Animal models that present with amyloidosis

The first attempts at generating transgenic models of AD based on the amyloid cascade hypothesis were performed by insertion of transgenes harboring wild-type human APP (hAPP) cDNAs. Of the initial three models, only one presented with consistent AD-like features [40]. Neuron-specific overexpression of the APP(751) isoform, but not the APP(695) variant, resulted in the formation of diffuse, pre-amyloid deposits of Aβ and rare mature, neuritic-like

plaques in murine cortex and hippocampus [41, 42]. After the identification of FAD-linked mutations in the APP and PS genes, several mouse models were designed that included both APP overexpression and one or more of these mutations. Two of these later models, the APP/PS1 and TgSwDI/B lines, were used for this work. APP/PS1 animals were selected because, unlike earlier models that develop robust amyloid deposition after 12 months of age, they initiate amyloid deposition at 6 months of age. TgSwDI/B animals were also used because they display early cerebrovascular deposition of amyloid, a prominent feature of AD neuropathology that is either absent or develops after more than 12 months in traditional murine models of AD.

1.2.3.1 APP/PS1 model

This model includes both the Swedish FAD-Linked mutation on a human APP gene and a mutant PS gene. Previous models had been engineered to express APP containing FAD-linked mutations alone. The rationale for the development of this mouse model included reports showing an increase in Aβ42/Aβ40 ratio in plasma and conditioned media from fibroblasts of PS1/PS2 mutation carriers, suggesting that PS mutations increased the generation of amyloidogenic Aβ42 peptides [43]. To test the impact of PS mutations on APP processing *in vivo,* Borchelt and colleagues developed several transgenic lines coexpressing APP and human PS1[44, 45].

The first report on this model included data from a line developed by co-expressing a chimeric Mo/Hu APP695 harboring the Swedish mutation and a human PS1 gene containing the A246E FAD-linked mutation [45]. Both transgenes were expressed individually from a murine prion promoter vector. These APP/PS1 animals confirmed the hypothesis that PS1 mutations elevate the ratio of Ab42/Aβ40 when compared to animals expressing APPSwe and/or wild-type PS1 alone. Furthermore, coexpression of APPSwe and PS1-A246E dramatically accelerated the development of hippocampal and cortical neuritic plaques when compared to mice expressing

either APPSwe or wild-type human PS1 alone [44]. A second line of bigenic animals was created by coexpression of the Mo/Hu APPSwe and the ΔE9 variant of the human PS1 gene, which results in deletion of exon 9 of the PS1 transcript. This line was used for all the studies presented herein. Both lines develop similar neuropathology and show age-related spatial memory deficits [46, 47]. Importantly, both APP and PS1 transgenes in the bigenic model have cointegrated into a single locus and are co-inherited as a single unit, a feature that greatly simplifies breeding strategies to other mice of interest.

1.2.3.2 TgSwDI/B model

Cerebral amyloid angiopathy (CAA) is a common feature of AD neuropathology caused by amyloid deposition in the cerebrovasculature, and is found in more than 80% of AD patients. The exact mechanism underlying the increased vascular accumulation of Aβ peptides remains unknown. To gain insights into this question, Davis and co-workers developed an APP transgenic mouse model designed to recapitulate the amyloid deposition pattern of AD with prominent CAA. For this, they generated a human APP(770) construct that contained the Swedish K670N/M671L, Dutch E693Q, and Iowa D694N mutations under the expression of the Thy 1.2 promoter [48]. Both Dutch and Iowa mutations reside within residues 21–23 of the Aβ domain of APP and preclude transport of Aβ across the blood brain barrier, resulting in familial forms of CAA [49-51].

TgSwDI/B animals exhibit modest overexpression of the hAPP transgene only in neuronal tissues and develop both parenchymal and vascular Aβ deposits starting at 3 months of age. The parenchymal deposits occur mainly in the subiculum, hippocampus and cortex and are of diffuse nature. Vascular Aβ deposits, on the other hand, are of fibrillar nature, displaying strong Thioflavin-S staining within the thalamic and subiculum brain regions [48]. In addition, these mice also display cerebral vascular cell loss, reactive glia associated with the vascular amyloid

deposits and increased levels of the inflammatory cytokines interleukin-1β and -6 [52]. To date, no behavioral phenotype has been described for this model.

1.3 Alzheimer Disease and the cholesterol connection.

Several lines of evidence, described in detail below, suggest that altered cholesterol metabolism may be playing a key role in the development of AD. Polymorphisms in *APOE*, the main lipid carrier in the CNS, are robust genetic risk factors for late-onset sporadic AD, which accounts for greater than 95% of AD cases. In addition, cellular cholesterol levels have been shown to shift the proteolytic processing of APP towards the generation of Aβ peptides. Furthermore, Aβ has been implicated as a modulator of neuronal cholesterol homeostasis. However, epidemiological observations, including reports of reduced risk of AD in patients taking statins and associations between hypercholesterolemia and increased risk of late-onset AD, have been proven contradictory. Nevertheless, they provide valuable incentive for further studies and will be discussed as well.

1.3.1 AD and apoE

ApoE constitutes the main apolipoprotein in brain parenchyma and CSF and has a clear role in peripheral lipid metabolism (reviewed in [53]). In 1993, apoE was discovered to be a genetic risk factor for AD, and still remains the only validated risk factor for late-onset AD today. In humans, apoE is present in three isoforms: E2, E3 and E4 [54-56]. The isoforms differ in their amino acid residues at position 112 and 158. ApoE3 is the most prevalent isoform, present in 70 to 78% of the general population, and exhibits a cysteine residue at position 112 and an arginine at residue 158. ApoE2 is the least prevalent variant (7-8 %) and displays cysteine residues at

both positions. Finally, apoE4 has a prevalence of 20 to 30% and contains arginines at both residues 112 and 158 (reviewed in [57]).

Initially examined because of its ability to bind Aβ in the CSF, studies on apoE and AD quickly revealed that the apoE4 isoform was overrepresented in AD patients [58]. Additional analyses showed a gene-dose effect of the apoE4 allele on both the likelihood of developing AD and the age of onset of the disease. Carriers of one apoE4 allele have a two- to five- fold increase in the risk of developing AD compared to non-carriers. This risk is increased above fivefold in carriers of two apoE4 alleles [7, 59, 60]. The apoE2 allele, on the other hand, is protective with respect to AD, as apoE2 carriers exhibit the lowest risk of developing AD [8.] Furthermore, the age of onset of AD drops from 84 years of age in non apoE4 carriers to 68 years of age in carriers of two copies of apoE4 [59].

Although several different roles in AD pathogenesis have been ascribed to apoE, the exact mechanism by which apoE influences the development of AD, in an isoform-specific manner, remains elusive. The most compelling evidence to date suggests that apoE is involved in Aβ clearance and/or deposition. ApoE has been shown to bind and form SDS-stable complexes with Aβ in an isoform specific manner, with E3 showing higher affinity for Aβ peptides than E4 [18, 61-63]. In addition, apoE has been implicated in the cellular uptake and degradation of Aβ by astrocytes and microglia [64, 65].

In vivo, AD transgenic, apoE-deficient animals are significantly impaired in their ability to form amyloid plaques and exhibit reduced Aβ immunoreactivity when compared to their apoE-expressing AD transgenic controls [17, 66-69]. Amyloid formation does eventually occur in apoE-deficient animals, but is present in less than 25% of apoE-deficient AD animals at 15 months of age, whereas at 12 months of age all apoE-expressing AD animals exhibit significant amyloid

pathology [70]. This demonstrates the requirement for apoE for the conversion of soluble Aβ to its fibrillar form. Interestingly, murine models of AD expressing human apoE3 and E4, in the absence of murine apoE, exhibit delayed amyloid deposition and neuritic plaque formation when compared to murine apoE-expressing controls [67, 71]. This suggests species-specific effects of apoE on Aβ metabolism. Finally, expression of human apoE2, E3 and E4 in the absence of endogenous murine apoE leads to isoform-specific differences in amyloid deposition, with E4>E3>E2 [71-74]. This last observation is consistent with the increase in vascular and parenchymal plaque Aβ deposits found in patients with sporadic AD expressing the apoE4 isoform versus apoE3 controls [75-79]. Although these data strongly implicate apoE in Aβ metabolism, the underlying mechanisms remain unknown. Factors that may influence the interaction of apoE with Aβ, including lipidation of this apolipoprotein, are thus of particular interest to study. This thesis work identifies ABCA1 as the first factor known to lipidate brain apoE *in vivo* and demonstrates that ABCA1-mediated lipidation of apoE has important consequences for amyloidogenesis.

1.3.2 APP processing and cholesterol

APP is a transmembrane protein and its proteolytic cleavage occurs both within and very closely to its transmembrane domain [25]. In addition, all the secretases are integral membrane bound proteins. *In vitro* studies using chemical or pharmacological methods to manipulate cholesterol have shown that high intracellular cholesterol concentrations increase Aβ production [80-82], whereas low cellular cholesterol levels reduce amyloidogenic processing of APP [83-87]. These data support the hypothesis that cellular cholesterol metabolism may be a key factor in the generation of Aβ and AD neuropathology.

ABCA1 induces cholesterol efflux from CNS-derived cells [10, 11] and, previous to 2004, three independent groups had implicated ABCA1 as a modulator of Aβ generation *in vitro*, with

conflicting results [10, 11, 80]. One study suggested that overexpression of ABCA1 increased secretion of Aβ42 [80], whereas two others observed decreased Aβ40 and Aβ42 levels in the presence of ABCA1 [10, 11]. However, because of the *in vitro* nature of these experiments, extrapolation of these results to whole organisms is difficult. This thesis provides evidence that absence of ABCA1 has no effect on Aβ generation, at least by measuring steady-state levels of soluble Aβ, but rather influences AD by modulating the function of apoE in amyloid formation.

Interestingly, Aβ itself has been implicated in cellular cholesterol metabolism as an inducer of cholesterol efflux [81], as a modulator of intracellular cholesterol esterification and distribution [82], and as modulator of cholesterol synthesis and sphingomyelin degradation [83, 90]. These observations have led some researchers to speculate that Aβ may be implicated in regulating cellular cholesterol metabolism. Further studies are needed, however, to test this hypothesis and to evaluate a possible role for Aβ in brain lipid homeostasis.

1.3.3 AD and plasma lipids

Several animal studies have consistently shown that diet-induced hypercholesterolemia increases Aβ generation [84-87]. Altered processing of APP due to increased cerebral cholesterol levels [85], and induction of a proinflammatory state in the cerebrovasculature [88] have been implicated as possible mechanisms underlying these effects. However, this association is less clear in humans. Clinical studies support a positive relationship between increased plasma cholesterol in mid-life and the development of dementia, but this association is less clear for AD [89-93]. Furthermore, data on the relationship between plasma lipid levels late in life and the risk for AD is controversial [94]. Large, prospective epidemiological studies clarifying this potential association are needed. This is of special importance if one considers the impact that inexpensive life-style changes may have on the epidemiology of AD.

1.3.4 AD and statins

Statins inhibit the enzymatic action of Hydroxymethylglutaryl CoA Reductase (HMG CoA Reductase), the enzyme responsible for the rate-limiting step in the synthesis of cholesterol. In 2000, two retrospective epidemiological studies showed dramatically reduced prevalence of AD in patients taking statins [95, 96]. As reviewed by Rockwood, subsequent cross-sectional studies confirmed these results. However, more recently, two large clinical trials and three longitudinal studies have concluded that there is no protective effect of statin use on cognitive decline and that data analysis methodology and design of previous studies may have introduced confounding factors that led to spurious results [97]. Similarly, mixed observations on the effects of statin treatment on AD neuropathology have been reported in animal models of AD [86, 98, 99]. A prophylactic or therapeutic role for statins remains thus controversial at this point. Further studies, especially large randomized, prospective, placebo controlled trials will be of great importance to determine the effectiveness of statin treatment in the prevention and treatment of AD, and to provide the rationale for further investigations into the mechanisms underlying such protection.

Taken together, these observations strongly suggests that proteins involved in lipid and lipoprotein metabolism, such as the cholesterol transporter ABCA1, may have key roles in the development and progress of AD.

1.4 ABCA1: a key player in HDL generation

ABCA1 is a member of the ATP-binding cassette (ABC) family of transporters and mediates the rate-limiting step of high-density lipoprotein (HDL) biogenesis. HDL is one of the four lipoprotein classes found in the peripheral circulation.

1.4.1 Lipoproteins: structure and function

Lipoproteins are water-soluble complexes of lipids and proteins, known as apolipoproteins, which transport hydrophobic lipids through aqueous body fluids such as plasma, interstitial fluid, lymph and cerebrospinal fluid (CSF). The function of lipoproteins is to deliver lipids from tissues and cells that synthesize them to those that use or store them.

The most studied pool of lipoproteins in the human body corresponds to plasma lipoproteins. The lipoproteins found in CSF, lymph or interstitial fluid are less well characterized. Plasma lipoproteins are classified based on their hydrated density into chylomicrons (CM), very low-density (VLDL), low-density (LDL) and high-density (HDL) lipoproteins. CSF lipoproteins, in turn, display densities of 1.10 to 1.21 g/ml, similar to plasma HDL particles [100, 101] (Figure 1-2). The density of each lipoprotein class is determined by their relative content of protein and lipids, and the diameters of these lipoprotein particles are inversely correlated with their densities, with chylomicrons measuring around 6000 Å and the smallest HDL particle about 70 Å. The diameters of the HDL-like particles found in human CSF range between the ones for plasma LDL and HDLs [100-103].

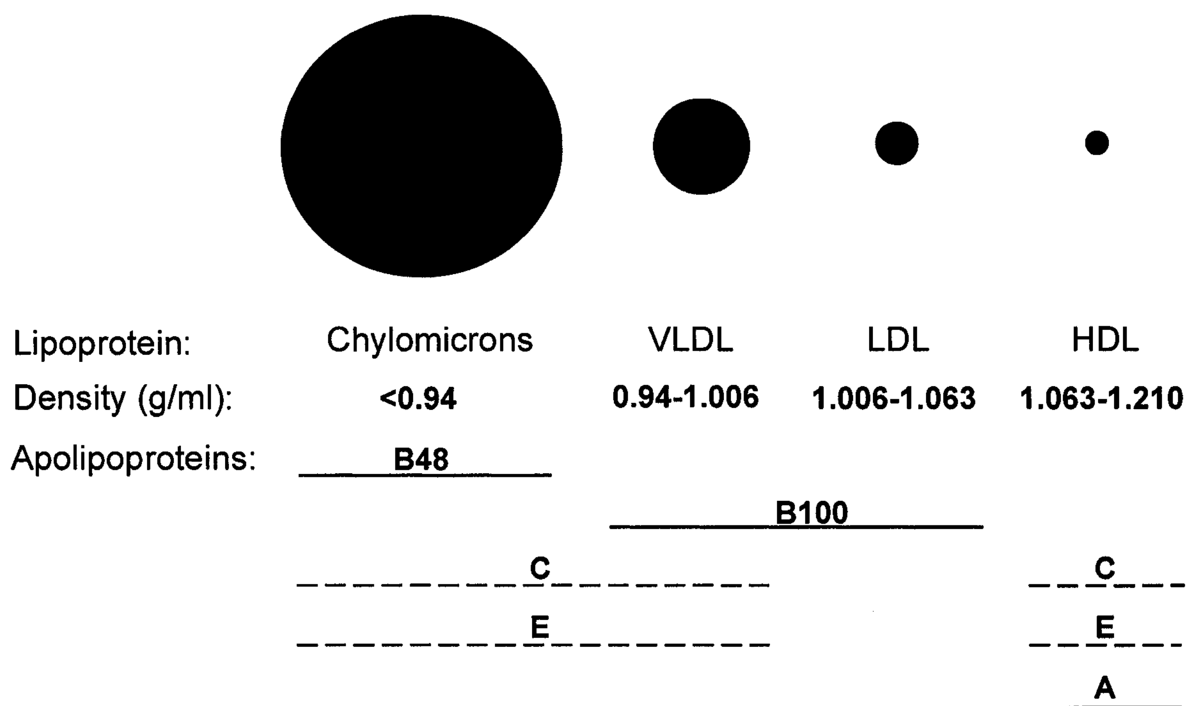

Figure 1-2: Schematic of the major lipoprotein classes based on size and density.
Solid lines indicate main apolipoprotein components. Dashed lines represent secondary or minor particle apolipoproteins.

All mature lipoproteins reveal the same basic structural features. They are spherical particles composed of a core of hydrophobic neutral lipids, mainly triacylglycerols and cholesterol esters, surrounded by a surface layer made up of relatively hydrophilic constituents such as amphipathic lipids (mostly phospholipids and unesterified cholesterol) and apolipoproteins [104]. There are several different apolipoproteins in the peripheral circulation. They are designated by letters and each one is found on distinct, sometimes overlapping, lipoprotein classes (Figure 1-2).

Although all lipoprotein classes share the same basic structure, differences in apolipoprotein and lipid composition result in distinct class-specific functions [104]. Chylomicrons, the most lipid-rich of all particles and the least dense ones, are synthesized in the intestine and specialize in the transport of dietary triacylglycerols to various tissues. VLDLs transport endogenous

triacylglycerols from the liver to other tissues, and LDL arise from the metabolic transformation of VLDLs in the circulation. The role of LDLs is to deliver cholesterol in the form of cholesterol esters to the liver and peripheral tissues. HDLs, the densest, smallest and most protein-rich of all lipoproteins, participate primarily in the removal of excess cholesterol from cells and transport it to the liver for excretion. This phenomenon is called 'Reverse Cholesterol Transport'. Recent evidence suggests that liver and intestine constitute the major sources of immature, phospholipidated HDL particles and that further acquisition of cholesterol occurs in extra hepatic tissues [105-108].

1.4.2 Physiology of ABCA1

ABC transporters, such as ABCA1, derive energy from ATP molecules to drive the transport of different substrates across cell membranes [109]. They are classified based on the presence, sequence and organization of their ATP-binding domains, also known as nucleotide-binding domains (NBD), and their transmembrane domains (TM). The NBD contains three conserved domains: Walker A and B domains and a signature (C) motif, the latter being unique to ABC transporters. A functional ABC transporter typically contains two TM domains and two NBDs (Figure 1-3). The eukaryotic ABC genes are organized either as full transporters, where all necessary domains are encoded in a single polypeptide chain, or as half transporters, which need to form homodimers or heterodimers with other half ABC-transporters to be functional (reviewed in [109, 110]). Among ABC transporters, ABCA proteins correspond to full transporters and have a 'classical' domain organization. Thus, they display six TM domains located N-terminal to the first NBD, followed by a second set of six TM domains and its corresponding NBD [111].

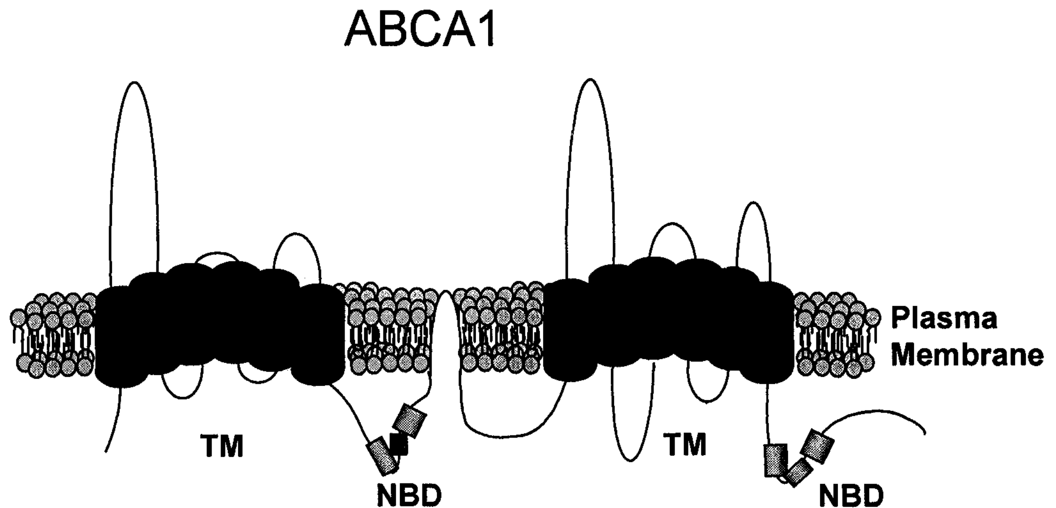

Figure 1-3: Diagrammatic representation of the ABCA1 protein.
TM=transmembrane domain; NBD= nucleotide binding domain.

ABCA1 is an integral membrane protein of 2,261 amino acids first identified as the product of the defective Tangier Disease (TD) gene [112-114]. TD patients present with low plasma HDL cholesterol, extremely low circulating apoA-I levels and cellular cholesterol accumulation, especially in the reticuloendothelial system, which results in hepatosplenomegaly and enlarged, grayish yellow or orange tonsils. Mutations in only one ABCA1 allele result in Familial Hypoalphalipoproteinemia (FHA), which is characterized by moderately reduced plasma HDL-C levels [14]. Together, these observations show that a primary biochemical function of ABCA1 is to regulate the metabolism of HDL particles. Indeed, ABCA1 transports cholesterol and phospholipids across the plasma membrane onto poorly lipidated apoA-I, thus initiating the formation of peripheral HDL (reviewed in [6]). ABCA1 transports lipids specifically onto lipid-poor apolipoproteins, including apolipoproteins AI, AII, E, CI, CII, CIII, and AIV [115, 116]. At least two working models for how ABCA1 effluxes lipids have been proposed. One model hypothesizes that direct binding of apoA-I to ABCA1 is required for the transfer of phospholipids and cholesterol onto the apolipoprotein [117-119]. A second model proposes that ABCA1 acts as a translocase flipping excess cholesterol and phospholipids from the cytosolic leaflet of the

plasma membrane to the external leaflet, generating a propitious membrane microenvironment for subsequent apoA-I docking and lipid efflux [6, 120, 121]. Finally, whether lipid efflux occurs at the plasma membrane, or also involves the internalization and resecretion of lipoproteins [122], is not yet fully understood.

ABCA1 is widely expressed in murine tissues, with high expression levels observed in liver, placenta, brain, testis, adrenal glands, and lung [9, 15]. It is interesting to note that ABCA1 protein levels do not always correlate with ABCA1 mRNA levels, as some tissues with high ABCA1 mRNA exhibit relative low levels of ABCA1 protein [9]. This is especially true in human atherosclerotic plaques, where, in spite of increased ABCA1 mRNA expression compared to normal arteries, protein levels have been reported to be significantly decreased [123]. This suggests that ABCA1 may be highly regulated at the posttranscriptional level.

The most studied activators of ABCA1 gene expression are agonists of liver X receptors and retinoic X receptors (LXR/RXR) [124, 125]. Oxysterols (LXR agonists) and retinoic acid (RXR agonist) exert their synergistic effects by inducing heterodimerization of LXRs with RXRs. These heterodimers then bind to response elements found within the promoter and first intron of the ABCA1 gene and increase its transcription [125-127]. LXR/RXR-independent factors that increase ABCA1 expression includes cAMP, the calcium channel blocker verapamil, inflammatory cytokines and other transcriptional response elements located on the ABCA1 gene promoter (reviewed in [6]).

Posttranscriptionally, ABCA1 levels are known to be modulated through protein stability. In the absence of apolipoproteins, phosphorylation of two threonine residues within a proline-glutamate-serine-threonine motif (PEST motif) occurs [128], which promotes ABCA1 proteolysis by an unknown member of the calpain protease family [128, 129]. This proteolysis occurs rapidly

resulting in a half-life of 1–2 h. In contrast, addition of apolipoproteins has been shown to stabilize ABCA1 in peripheral cells and liver [130, 131].

Phenotypes for both ABCA1-deficient and overexpressing animal models have been reported. Like TD patients, ABCA1-deficient animals present with dramatically decreased total- and HDL-cholesterol levels, hypercatabolism of apoA-I and accumulation of cholesteryl esters in several tissues, especially in those rich in macrophages [108, 132, 133]. Conversely, ABCA1-overexpressing mice present with elevated plasma HDL and circulating apoA-I levels, and are protected against macrophage lipid accumulation [106, 134-136].

Together, these data clearly demonstrate the pivotal role of ABCA1 in HDL biogenesis and apoA-I metabolism outside the brain. Although ABCA1 expression had been reported in the brain, little was known about its role in CNS cholesterol and lipoprotein metabolism. This work provides one of the first observations supporting a key role for ABCA1 in the maintenance of lipid homeostasis in the CNS through its modulation of apoE levels and lipidation.

1.5 Cholesterol and lipoprotein metabolism in the Central Nervous System

Compared to peripheral lipoproteins, the role of brain lipoproteins in lipid homeostasis in the central nervous system (CNS) is much less clear. Brain lipoproteins are thought to play a key role in shuttling cholesterol between glial cells that synthesize and secrete lipoproteins and neurons that rely on the uptake of exogenous cholesterol to meet their requirements for membrane biosynthesis. However, the identities of the proteins involved in brain lipid metabolism have just begun to be revealed. This thesis provides evidence supporting the hypothesis that proteins involved in peripheral lipoprotein metabolism may function in a similar manner in the CNS.

hypothesis that proteins involved in peripheral lipoprotein metabolism may function in a similar manner in the CNS.

In the brain, more than 99.5% of the cholesterol is unesterified and is found in insulating myelin sheets and cell membranes of glia and neurons (reviewed in [2, 137]). To maintain optimal levels of cholesterol in their membranes, cells in different organs make use of two mechanisms for its acquisition: *de novo* synthesis or uptake of cholesterol-containing lipoprotein particles from the circulation. Several lines of evidence strongly suggest that the majority of cholesterol accumulated within the CNS derives from synthesis *in situ*. First, the synthesis rate of cholesterol in the brains of rats and lambs, as measured by the incorporation of radioactivity from peripherally injected [3H]water, is able to cover the requirements for cholesterol during early neonatal development and adulthood [138, 139]. Second, peripheral administration of radioactive cholesterol results in isotope labeling of all tissues except brain [140-142]. Third, no detectable radioactivity is found in the brain after injection of isotope labelled LDL or HDL and deletion of lipoprotein receptors in whole animals does not alter cholesterol content or synthesis in the brain [143-146]. Together, these observations demonstrate that the overwhelming majority of CNS sterols derive from local synthesis.

Although sterol synthesis has been shown to occur in each cell type within the brain, some authors hypothesize that glia are the primary producers of cholesterol and that delivery of this sterol to neurons occurs via glial-derived lipoproteins (reviewed in[147]). This hypothesis is based on several observations. First, in a strict glia-free *in vitro* system, primary retinal ganglion neurons require the presence of cholesterol-containing lipoproteins derived from astrocytes to form mature excitatory synapses [148]. Furthermore, axonal growth of CNS-derived primary neurons is significantly promoted by the presence of cholesterol and apolipoprotein E *in vitro* [149]. And though the requirement for glia-derived lipoproteins for normal neuronal development has not been demonstrated *in vivo*, it is interesting to note that most synapses develop after

astrocytes have differentiated [150, 151]. Astrocytes are also able to synthesize two to five fold more cholesterol than neurons *in vitro* [152, 153] and secrete cholesterol-containing lipoprotein particles [153-156]. Finally, neurons express cellular receptors for these particles [101, 158-161] and the neuronal binding capacity for iodinated LDL increases dramatically during periods of high cholesterol requirements such as reinnervation [3]. Taken together, these data strongly support the hypothesis that CNS cholesterol is synthesized and secreted predominantly by glia and then circulates on glia-derived lipoproteins within the brain where their availability to neurons becomes particularly important during periods of high lipid demand such as synaptogenesis or neurite repair.

Unlike CNS cholesterol that is entirely synthesized within the brain, CNS apolipoproteins can originate locally or be derived from the peripheral circulation. Apolipoproteins A-I, A-II, A-IV, C-I, C-II, C-III, D, E and J have been described in the CSF of several species, mostly in the form of lipoproteins [100-103]. Of these, apoE and apoA-I correspond to the main apolipoproteins found in the CSF at approximately 4% and 0.5% of their plasma concentration, respectively [100, 101]. Interestingly, *in situ* hybridization studies have shown mRNA expression of apolipoproteins C-I, D, E and J in the CNS (reviewed in [161]). This suggests that the latter apolipoproteins are synthesized locally, whereas the rest of the CSF apolipoproteins, including apoA-I and apoA-IV, are imported from the peripheral circulation. However, the role of endogenous vs. peripheral apolipoproteins in CNS lipid metabolism remains to be fully elucidated. Furthermore, the question whether ABCA1 is involved in the generation of CSF HDL-like particles was unclear. Importantly, apoB is not found within the brain or CSF, indicating that brain lipoprotein metabolism revolves around particles that resemble HDLs.

The brain is a major site of synthesis for both apoE and apoJ. ApoE is a ubiquitously expressed glycoprotein of 299 amino acids and the CNS constitutes the second major site of apoE synthesis after liver [162]. Within the brain, apoE mRNA has been localized by in situ hybridization

mainly to astrocytes [162-164]. Accordingly, cell culture experiments have shown that glial cells are capable of apoE synthesis and secretion [5, 155]. Nascent immature glia-derived apoE particles are discoidal in shape and poorly lipidated, whereas mature CSF apoE particles are spherical and fully lipidated [154, 165].

Several receptors for apoE have been described in the CNS on neuronal and non-neuronal cells. They are all members of the LDL receptor family of proteins and include the LDL-Receptor (LDLR), LDL-Receptor Related Protein (LRP), VLDL-Receptor (VLDLR), gp330/megalin, and the recently described apoE Receptor 2 (apoER2) (reviewed in [161]). LDLR-deficient mice demonstrate a significant increase in brain levels of both mouse apoE and human apoE3 and apoE4, but not apoE2, protein levels [166]. This is probably due to the lower binding affinity to LDLR exhibited by apoE2, which impairs efficient internalization of apoE2. ApoE2 levels are thus already maximal in the absence of the LDLR. Although the LDLR has been shown to mediate the vast majority of the binding and internalization of glial-derived apoE in cellular models [166], other apoE receptors may also play a role *in vivo*. For example, LDL-Receptor Related Protein-Associated Protein (RAP)-deficient mice, which exhibit a 75% and 40% reduction in LRP and LDLR levels, respectively, show a significant decrease in brain apoE protein [167]. In addition, a 3.7-fold overexpression of murine LRP does not alter brain apoE levels [168]. Together, these *in vivo* studies suggest a complex network of interactions between apoE receptors and the regulation of apoE levels in the brain.

CNS apoE, including CSF apoE, is thought to derive solely from apoE synthesized in the brain. This conclusion derives from observations in liver transplant patients where the donor and recipient differ in terms of apoE genotype. In these patients, most plasma apoE is converted to the donor's variant, indicating that the transplanted liver takes over synthesis of plasma apoE. In contrast, the identity of the CSF apoE isoforms remains unchanged after liver transplantation. This illustrates that CNS and non-CNS apoE pools are synthesized

independently and that peripheral apoE is not imported into the CSF [169]. This last observation is further supported by experiments in guinea pigs that show very low transport of peripherally-administrated radiolabelled apoE across the blood-brain-barrier (BBB) [170].

Although some studies report age-dependent cognitive deficits [171-173], cholinergic dysfunction [171, 172] and synaptic loss [174, 175] in normally aged apoE-deficient animals compared to wild-type controls, other analyses have not found differences between these two groups [176-178]. However, different brain lesion paradigms indicate that apoE-deficient mice exhibit greater cognitive and neuronal dysfunction compared to controls [179-181]. Interestingly, Alzheimer transgenic animals on an apoE-deficient background display greater object recognition impairments than apoE-deficient or AD transgenic controls [182]. This suggests that under physiological conditions, apoE-independent pathways compensate for the absence of CNS apoE. However, when stressed by injury, these alternate mechanisms are no longer able to balance the apoE deficiency, leading to reduced synaptogenesis and neuronal dysfunction.

ApoJ is a multifunctional protein of 449 amino acids (also known as clusterin, SP-40,40 or SGP-2) that has been implicated in lipid transport, sperm maturation, complement inhibition, tissue remodeling, intracellular vesicular packaging, cellular adhesion and migration and is thought to have chaperone-like activity (reviewed in [183]). Similar to apoE-/- mice, apoJ-deficient mice do not display gross morphological or histological abnormalities, both male and female homozygous deficient animals are fertile, and females give birth to normal-size litters [184]. In *situ* hybridization studies have demonstrated the presence of apoJ mRNA in neuronal and non-neuronal cells, especially in astrocytes in the vicinity of blood vessels (reviewed in [161]). Unlike apoE, however, apoJ has shown ample transport across the BBB [170]. One of the questions that remain to be addressed is whether these brain apolipoproteins participate in CNS and peripheral lipoprotein metabolism in a similar mode.

1.6 ABCA1 in the brain

ABCA1 has been shown to be expressed in glial and neuronal cells, particularly in Purkinje cells and pyramidal cortical neurons [9, 10, 185], as well as in cultured brain capillary endothelial cells [186]. LXR/RXR ligands induce ABCA1 expression in CNS-derived cells and increase cholesterol efflux to apoA-I and apoE [10, 11]. Because ABCA1 regulates intracellular cholesterol levels it is plausible to hypothesize that modulation of ABCA1 expression may affect APP processing. Indeed, three *in vitro* studies have evaluated whether ABCA1 overexpression modulates Aβ generation yielding conflicting results. One study showed that overexpression of ABCA1 increased secretion of Aβ42 [80], whereas two others suggested the opposite effect [11, 12]. The reason for this discrepancy has not been addressed, but it serves to highlight the importance of evaluating the effect of ABCA1 on APP processing in an *in vivo* model. In this thesis, data is provided that suggests that absence of ABCA1 does not impact APP processing and Aβ generation *in vivo*, but may alter the development of AD neuropathology by modulating the role of apoE in amyloidogenesis.

Finally, genetic studies have also generated contradictory results on the association between AD and genetic variations of ABCA1. Two studies have reported that ABCA1 genetic variations modify the risk for Alzheimer's disease [187, 188], whereas two other case-control studies found no such relationship [189, 190]

1.7 Summary, hypothesis and specific objectives

Strong experimental data supports a causative role for Aβ in AD. Cholesterol modulates the production and deposition of Aβ. Cellular cholesterol levels may determine the generation of toxic Aβ peptides, and unknown apoE isoform-dependent variations in brain cholesterol trafficking may constitute one of the greatest risk factors for late-onset AD. Of note, apoE has been implicated in a variety of functions in the CNS, but the question whether lipidation of apoE

may alter these functions *in vivo* remained unanswered. In this thesis we present novel *in vivo* data supporting a key role for ABCA1 in the maintenance of brain apoE levels and lipidation. We also report that lipidation status of apoE significantly affects the function of this apolipoprotein, further confirming a role for lipid metabolism in AD.

In summary, the cholesterol transporter ABCA1 is expressed in the brain, modulates cellular cholesterol levels, and determines secretion and lipidation of apoE in the periphery. However, a more detailed model of ABCA1's function in the CNS had not been postulated. To test the hypothesis that ABCA1 affects apolipoprotein metabolism *in vivo*, and thus influences AD neuropathology, we have made use of well validated murine models for ABCA1-deficienciency and overexpression, as well as AD models that recapitulate many of AD's neuropathological hallmarks. Thus, the main hypothesis driving this work is that **ABCA1 plays a key role in the development of AD neuropathology and in brain lipid metabolism.** This question was addressed in three specific aims:

- Aim 1: To evaluate the impact of ABCA1 deficiency on brain apoE and cholesterol metabolism.
- Aim 2: To assess the effect of ABCA1 deficiency on the development of AD neuropathology.
- Aim 3: To determine the significance of ABCA1 overexpression on both brain apoE metabolism and AD neuropathology.

The relevance of this thesis work lies in that it establishes that ABCA1 is required for the maintenance of normal levels of brain apoE and provides novel data suggesting that, *in vivo*, apoE function is modulated by its lipidation status. In addition, it presents partial insights into the mode of action of LXR agonists, which have shown promising, but preliminary, therapeutic benefits in murine models of AD.

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Chapter 2 - Deficiency of ABCA1 impairs apolipoprotein E metabolism in brain*

2.1 Introduction

ABCA1 is a member of the ATP-binding cassette superfamily of transporters that transport substrates across membranes [1-3]. A major biochemical function of ABCA1 is to transport cholesterol and phospholipids from the plasma membrane of peripheral cells to lipid-free or lipid-poor apolipoprotein A-I (apoA-I) during reverse cholesterol transport [1], a process that constitutes an initial step in the biogenesis of high-density-lipoproteins (HDL) in the peripheral circulation. Mutation of one ABCA1 allele causes familial hypoalphalipoproteinemia (FHA), a mild disorder of lipid metabolism characterized by reduced plasma HDL levels [4]. Mutation of both alleles of ABCA1 results in Tangier Disease (TD), which is characterized by a nearly complete absence of plasma HDL, deposition of intracellular cholesterol esters, and an increased risk of cardiovascular disease [4-6]. Conversely, overexpression of ABCA1 in mice increases plasma HDL and protects against atherosclerosis [7-9].

ABCA1 is abundant in liver and macrophages, where its expression is induced by agonists of the liver X receptor/retinoid X receptor (LXR/RXR) pathways [10-16]. ABCA1 is also expressed in both glia and neurons in the central nervous system (CNS), where it is particularly abundant in Purkinje cells and in large pyramidal cortical neurons [10, 17, 18]. Similar to non-CNS cells, ABCA1 is induced by LXR/RXR stimulation in primary neurons, astrocytes, and microglia [18-20].

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The importance of lipid homeostasis in the CNS is underscored by the fact that the brain is the most cholesterol-rich organ in the body, and contains approximately 25% of total body cholesterol in only 2% of the total body weight [21]. Nearly all brain cholesterol is synthesized in situ, as quantitative analyses show that essentially no cholesterol crosses the blood-brain-barrier [21]. In contrast, the brain cannot degrade cholesterol, and excess cholesterol must be delivered to the peripheral circulation for eventual hepatic excretion. Approximately 6-7 mg of cholesterol leaves the human brain each day after conversion to 24S-hydroxycholesterol, which easily traverses the blood-brain-barrier [22, 23].

Glial cells play crucial roles in regulating lipid homeostasis in the CNS. Astrocytes and microglia are the cells within the CNS that synthesize and secrete apolipoproteins that transport lipids within the brain and cerebrospinal fluid (CSF) [24-27]. Apolipoprotein E (apoE) is the major apolipoprotein in the brain, and is also a component of several lipoproteins in the peripheral circulation including HDL, very low density lipoproteins (VLDL), and chylomicrons [28-30]. In humans, apoE exists as one of three allelic variants that contain either a cysteine or arginine residue at amino acids 112 and 158 [31]. Liver transplantation experiments in humans have shown that the allelic variant of apoE in plasma is converted to that of the donor, whereas apoE in the CSF retains the allelic identity of the recipient, illustrating that CNS and non-CNS apoE pools are synthesized independently [32]. Additionally, brain perfusion experiments in guinea pigs have shown that radiolabelled apoE administered into the peripheral circulation demonstrates very low blood-to-brain transport [33], providing additional support for independent regulation of CNS and non-CNS apoE pools. ApoJ is a second major CNS lipoprotein that, unlike apoE, easily traverses the blood-brain barrier and associates with HDL in the periphery [26, 33, 34]. ApoE and apoJ are synthesized and secreted from astrocytes and microglia, and expression of both apoE and apoJ are induced in response to CNS injury or disease [24, 26]. ApoE is thought to coordinate the mobilization and redistribution of cholesterol in the repair and

maintenance of neuronal membranes and myelin [35-37], whereas apoJ may function as a cytoprotective extracellular chaperone [38]. In contrast to apoE and apoJ, apoA-I is primarily taken up into the brain from outside the CNS, and is believed to be involved in the maturation of lipoprotein particles in the CSF [26, 39].

In 1993, apoE was discovered to be an important genetic risk factor for Alzheimer's disease (AD), a finding that has now been replicated in over 100 studies. Inheritance of apoE4 increases the risk of AD in a dosage specific manner and may act by decreasing the age of onset of AD [40, 41]. In contrast, inheritance of apoE2 appears to protect from AD and is associated with a later age of onset [42]. Many hypotheses have been proposed to explain how apoE participates in the pathogenesis of AD, including roles for apoE in the formation, deposition, or clearance of the Aβ peptides that constitute amyloid plaques [43-51]. For example, apoE binds Aβ *in vivo* and is required for amyloid deposition [50-53]. Furthermore, apoE affects deposition of Aβ in an isoform-specific manner, such that mice expressing human apoE4 develop at least 2.5 fold more Aβ deposits than mice expressing human apoE3 per unit area [49, 53-55]. Interestingly, replacement of the murine apoE with any of the human apoE isoforms significantly delays Aβ deposition compared to wild-type mice expressing endogenous murine apoE [49, 56], suggesting that distinctions between human and murine apoE may play important roles in Aβ deposition and/or clearance. Compared to apoE, the role of apoJ in AD is less well understood, although it is known that apoJ also binds Aβ and has been suggested to play a major role in transporting Aβ across the blood-brain barrier [33, 34]. In addition, *in vivo* experiments suggest that apoJ plays an important role in amyloidogenesis. AD transgenic mice that are deficient in apoJ exhibit similar levels of brain Aβ deposition as their apoJ-expressing controls. However, and although Aβ levels are similar, apoJ-deficient animals have significantly fewer amyloid deposits than mice expressing apoJ [57].

Both ABCA1 and apoE have been shown to be induced in response to LXR/RXR stimulation [15, 16, 58, 59] and to be repressed by ZN202 [60] in peripheral macrophages. Previous work has demonstrated that apoE is a robust acceptor of lipids from ABCA1 [61, 62], raising the question of whether ABCA1-mediated lipid efflux may play important roles in the brain where apoE is a predominant apolipoprotein. Furthermore, antisense inhibition of ABCA1 has been observed to inhibit the secretion of apoE from monocyte-derived macrophages [63], raising a second question of whether ABCA1 may also regulate apoE synthesis or secretion in other apoE-producing cells such as astrocytes or microglia.

To address these two questions, we sought to determine the relationship between ABCA1, cholesterol efflux and apoE metabolism specifically in glia. In this study, we report that ABCA1-deficient primary astrocytes and microglia are impaired in their ability to efflux lipids to exogenous apoE, which results in lipid accumulation under normal culture conditions. Furthermore, lack of ABCA1 expression decreases apoE secretion from glial cells *in vitro*. *In vivo*, this impairment of apoE secretion by astrocytes and microglia results in a decrease in apoE levels by approximately 65% in whole brain lysates of ABCA1-deficient mice, with hippocampus and striatum being the most severely affected regions. Our observations show that ABCA1 plays key roles in cholesterol transport and apoE metabolism in the CNS.

2.2 Experimental procedures

2.2.1 Animals

ABCA1-deficient mice were generously provided by Dr. Omar Francone of Pfizer Global Research and Development (Groton). Wild-type DBA1/J, C57Bl/6, apoE-deficient and apoA-I-deficient mice were obtained from Jackson Laboratories. Animals were maintained on regular chow (PMI Lab Diet) for all experiments. All procedures involving experimental animals were

performed in accordance with protocols from the Canadian Council of Animal Care and the University of British Columbia Animal Care Committees.

2.2.2 Animal tissues

Murine brains were harvested immediately after sacrifice, dissected into individual regions where indicated, frozen on dry ice and stored at -80 °C until use. Tissues were homogenized in a buffer containing 10% glycerol, 1% TritonX-100 and one tablet of protease inhibitor (Roche) in phosphate buffered saline (PBS). Protein concentration was determined by Lowry assay.

2.2.3 Culture of primary astrocytes and microglia

Primary mixed glial cultures were prepared from postnatal day 1-2 mice. Brains from individual animals were placed into ice-cold Hanks Buffered Salt Solution (Canadian Life Technologies) containing 6 mg/ml glucose and 10 mM HEPES. Meninges were removed, frontal cortices were dissected and cells were dissociated by repeated passage through a series of wide to fine bored pipettes. Dissociated cells were plated in Dulbecco's Modified Eagle Medium (DMEM) (Canadian Life Technologies) with 10% fetal bovine serum (FBS), 10% horse serum, 2 mM L-glutamine (Canadian Life Technologies) and 100 units/ml of penicillin-streptomycin (Canadian Life Technologies) at one T75 flask per mouse. Cells were cultured in the presence of 5% CO_2 for 14 days with 3 medium changes. After reaching confluence, superficial microglia were separated from the glial monolayer by gently tapping the culture flasks and collecting the medium with detached cells. Subsequently, attached microglia were separated from astrocytes by mild trypsinization as previously described [64], and added to the previously isolated cells. Microglia and enriched astrocytes were reseeded as needed.

2.2.4 Immunofluorescence

Cells seeded on poly-D-lysine-coated coverslips were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature, washed twice more, and permeabilized with 0.5% Triton X-100 in PBS for 5 minutes at room temperature. After washing three times in PBS, cells were blocked with 4% normal goat serum in PBS for 30 minutes at room temperature, and stained with the astrocyte marker GFAP-Cy3 (1:250, Sigma) or the microglial marker F4/80 (1:200, Serotec) for 1 h. F4/80-stained cells were visualized after incubation with Alexa-488 conjugated secondary antibody (Transduction Laboratories) for 30 min. Finally, cell nuclei were stained with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI), and coverslips were mounted with Vectashield (Vector Laboratories). The percentage of stained cells was determined over at least 200 randomly selected cells. Cells were viewed under a Zeiss Axioplan 2 microscope and images were captured using a CCD camera equipped with Metamorph (Universal Imaging Corporation) imaging software.

2.2.5 Western blotting

Equal amounts of protein were electrophoresed through 7.5% or 10% SDS polyacrylamide gels, electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane (Millipore) and immunodetected using a monoclonal anti-ABCA1 antibody raised against the second nucleotide binding domain (NBD2 of ABCA1) [10], a murine-specific apoE antibody (Santa Cruz, 1:500), a polyclonal antibody against murine apoJ (1:250, generously provided by Dr. David Holtzman, Washington University School of Medicine) or an anti-GAPDH antibody (Chemicon, 1:10,000) as a loading control. Blots were developed using enhanced chemiluminescence (Amersham) according to the manufacturer's recommendations. Bands were quantitated by densitometry using NIH Image J. Where applicable, apoE, apoJ, and ABCA1 levels were normalized to GAPDH levels to control for protein loading.

2.2.6 Purification of recombinant apoE proteins

Recombinant human apoE isoforms were purified from *Escherichia coli* harboring recombinant glutathione-S-transferase (GST)-apoE2, -apoE3, or apoE4 fusion proteins as described [65]. Briefly, logarithmic-phase cells were induced with 0.5 mM IPTG for 1 h, harvested by centrifugation, lysed by sonication in 1.0% Triton X-100, 10% glycerol in PBS, 200 µg/ml lysozyme, and cleared by centrifugation. GST-apoE fusion proteins in the cleared lysates were purified over glutathione sepharose 4B beads and eluted upon cleavage of the GST moiety by Precission Protease (Amersham). Where indicated, purified apoE proteins were delipidated in 6M urea followed by dialysis against several changes of PBS.

2.2.7 Cholesterol efflux

Microglia and enriched astrocytes were seeded at 50,000 cells/well in 96 well-plates and labelled with 1 µCi/ml of 3H-cholesterol (New England Nuclear) for 18 to 24 h. Labelled cells were then washed and equilibrated in DMEM/F12 for 1 h, after which 10 µg/ml of lipid-free apoA-I (Calbiochem), or recombinant apoE2, apoE3, apoE4 were added for 8 h. Media was collected and centrifuged at 2000 rpm for 5 minutes. Cells were lysed by addition of 50 µl of 0.1 M NaOH and 0.2% SDS followed by incubation at room temperature for 20 minutes. 50 µl of media and cell lysate was added to scintillation vials and quantified. Percent cholesterol efflux was calculated as the total counts in the medium divided by the sum of the counts in the medium plus the cell lysate, as previously described [66].

2.2.8 Lipid staining

Cells were seeded in 24-well plates on poly-D-lysine-coated coverslips at 100,000 cells/well for microglia and 200,000 cells/well for astrocytes. After at least 18 h in DMEM containing 10% FBS and 10% horse serum, cells were air dried, fixed in neutral-buffered formalin at room

temperature and stained with Oil-red-O. Nuclei were counterstained with haematoxylin. Cells were photographed on a Zeiss Axioplan 2 microscope using a CCD camera equipped with Metamorph (Universal Imaging Corporation) imaging software.

2.2.9 Measurement of apolipoprotein secretion

Microglia and astrocytes were seeded in 96-well plates at 50,000 cells/well and 100,000 cells/well, respectively. After 18 h, cells were washed once with PBS and cultured in serum-free DMEM/F12 (Canadian Life Technologies) for 6 h (microglia) or 8 h (astrocytes). Conditioned medium was collected, centrifuged at 2,000 rpm for 4 min and the supernatant was stored at -80 °C until use. Cells were scraped, lysed in a buffer containing 10% glycerol, 1% Triton X-100 and protease inhibitor (Roche) in PBS and centrifuged at 9,000 rpm for 10 min. The supernatant was stored at -80 °C until use. Media and cell lysate samples were analyzed for apoE, apoJ, ABCA1, and GAPDH expression by Western blot as described above. Intracellular GAPDH levels were used to normalize measurements of cellular or secreted apoE.

2.2.10 Statistical analysis

One-way ANOVA with a Newman-Keuls post test or two-tailed unpaired Student's *t*-tests were used for statistical analysis. In the T-test analyses, Welch's correction for unequal variances was applied when variances were significantly different between groups. Where indicated, apoE and ABCA1 levels were correlated using linear regression analyses. All statistical analyses were performed using Graphpad Prism (version 3.0; Graphpad Software for Science Inc., San Diego, CA).

2.3 Results

2.3.1 Glial ABCA1 mediates cholesterol efflux to apoA-I and facilitates efflux to apoE

Several studies in non-CNS cells have established that ABCA1 promotes cholesterol and phospholipid efflux to lipid-deficient apolipoproteins, including apoA-I, apoE and others [61, 67, 68]. Whether ABCA1 functions similarly in CNS cells is not yet completely understood. Previous work has shown that ABCA1 is expressed in glia and neurons in the brain [10, 17] and that LXR/RXR stimulation of primary glia promotes cholesterol efflux to apoA-I and apoE3 [18]. However, the specific role of ABCA1 in promoting cholesterol efflux from glial cells is not yet clear because expression of other genes in addition to ABCA1 are also induced by LXR/RXR treatment [13, 58, 69]. To selectively address the role of ABCA1 in cholesterol efflux from glial cells, primary cultures of astrocytes and microglia were prepared from ABCA1-/- mice and wild-type littermate controls and were maintained in the absence of LXR/RXR treatment or cholesterol loading. The presence of ABCA1 protein in wild-type but not ABCA1-/- cultures was first confirmed using immunoblotting (Figure 2-1A). The purity of the astrocyte and microglial cultures used in these experiments was evaluated by immunofluorescent staining using anti-GFAP and anti-F4/80 to specifically label astrocytes and microglia, respectively. Experimental cultures were at least >85% pure for astrocytes and >98% pure for microglia (data not shown).

The role of glial ABCA1 in cholesterol efflux to exogenous apoA-I and apoE isoforms (apoE2, apoE3 and apoE4) was determined in primary wild-type and ABCA1-/- astrocytes and microglia. Cholesterol efflux was measured in the absence of apolipoprotein acceptors or in the presence of recombinant human apoA-I, apoE2, apoE3 or apoE4 for 8 h. Because preliminary experiments in mixed glial cultures showed no difference in cholesterol efflux in the presence of either native or delipidated apoE3, native apoE proteins were used for all subsequent experiments.

The effect of different apolipoprotein acceptors on cholesterol efflux from wild-type and ABCA1-/- astrocytes and microglia are illustrated in Figure 2-1 and summarized in Table 2-1. As expected, ABCA1 is required for astrocytes to efflux cholesterol to exogenous apoA-I. Under our experimental conditions, wild-type astrocytes displayed a 2.5 fold increase in cholesterol efflux upon apoA-I addition, whereas ABCA1-/- astrocytes did not show higher efflux to apoA-I compared to baseline ($p>0.05$) (Figure 2-1B, Table 2-1). In wild-type astrocytes, addition of apoE2, apoE3, and apoE4 resulted in a 2.3 – 2.6 fold increase in cholesterol efflux, which is similar in magnitude to that elicited by apoA-I. In contrast, the cholesterol efflux elicited by each apoE isoform was approximately 30% less in ABCA1-/- cells compared to wild-type astrocytes when corrected for differences in baseline efflux (Table 2-1). These observations suggest that ABCA1 in astrocytes facilitates lipid efflux to exogenous apoE. However, ABCA1 is not absolutely required to efflux cholesterol to apoE, as residual efflux activity between 1.5 - 1.8 fold remained in the absence of ABCA1. These observations suggest that genes in addition to ABCA1 also contribute to cholesterol efflux from astrocytes. Comparison among apoE isoforms suggested that apoE2 was a less effective cholesterol acceptor than apoE3 or apoE4 for both wild-type and ABCA1-/- astrocytes, whereas no significant differences were observed in efflux to apoE3 and apoE4 (Figure 2-1B). Compared to wild-type astrocytes, ABCA1-/- astrocytes were significantly impaired in their ability to efflux cholesterol to apoA-I and each apoE isoform ($p<0.001$, Table 2-1).

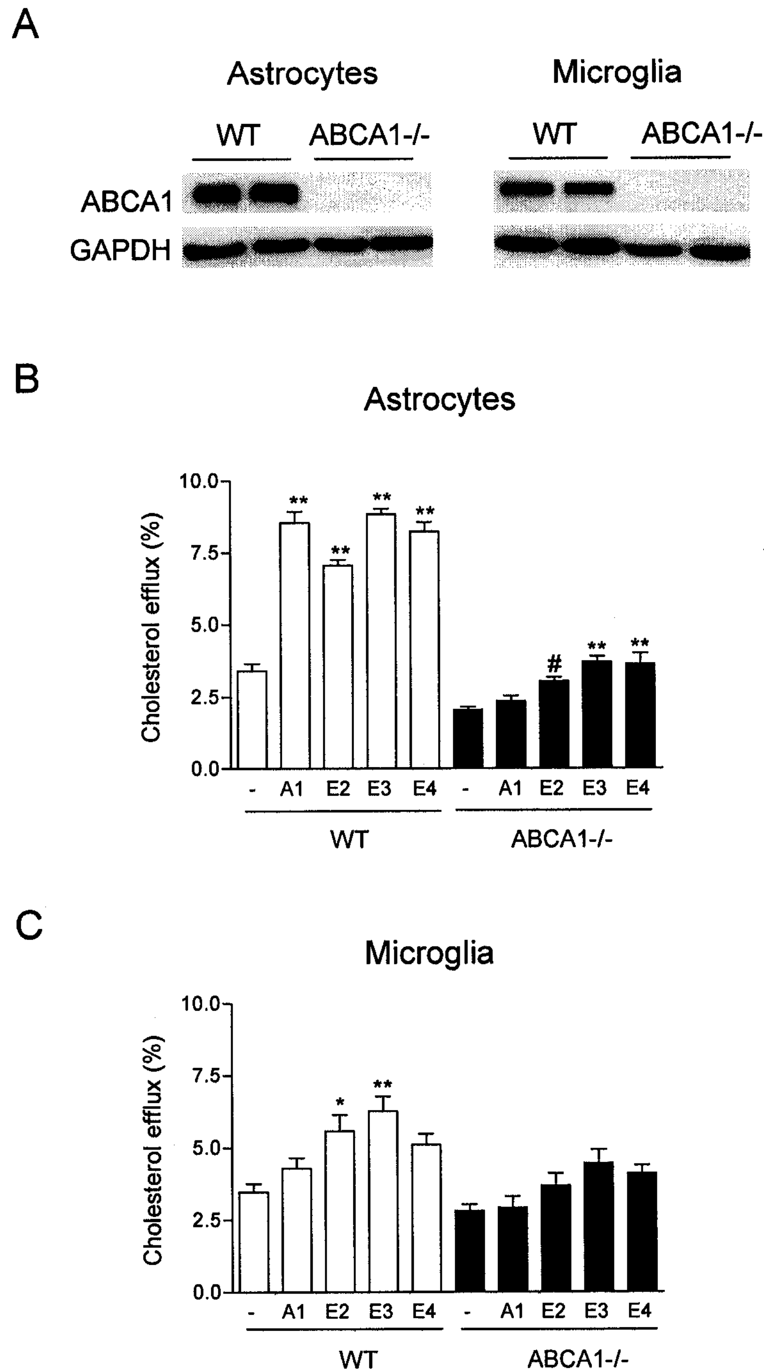


Figure 2-1: Cholesterol efflux is impaired in ABCA1-/- glia cells.

(A) ABCA1 expression in wild-type and ABCA1-/- primary astrocytes (left) and microglia (right) was first determined by western blotting using an ABCA1-specific antibody. GAPDH (lower panels) served to control for equal protein loading. Efflux for eight hours in the presence or absence of 10 μg/ml of exogenous apoA1, apoE2, apoE3 and apoE4 was evaluated in wild-type and ABCA1-/- astrocytes (B) and microglia (C). Graphs represent means and standard errors of at least two independent experiments with at least 6 individual mice per genotype. One-way ANOVA with a Newman-Keuls post test was used to determine significant increases in cholesterol efflux over baseline. (# represents $p<0.05$, * represents $p<0.01$, and ** represents $p<0.001$).

Table 2-1: Cholesterol efflux by different apolipoproteins in WT and ABCA1-/- glia

Cell type	Acceptor	Fold Efflux wild-type (*)	Fold Efflux ABCA1-/- (*)	% Reduction in fold efflux (**)	p value
Astrocytes	A1	2.5 +/- 0.23	1.1 +/- 0.23	55%	< 0.001
	E2	2.3 +/- 0.20	1.5 +/- 0.17	35%	< 0.001
	E3	2.6 +/- 0.20	1.8 +/- 0.18	30%	< 0.001
	E4	2.4 +/- 0.22	1.7 +/- 0.28	30%	< 0.001
Microglia	A1	1.2 +/- 0.31	1.0 +/- 0.40	15%	n.s
	E2	1.6 +/- 0.55	1.3 +/- 0.35	18%	< 0.05
	E3	1.8 +/- 0.30	1.6 +/- 0.34	11%	< 0.05
	E4	1.5 +/- 0.28	1.5 +/- 0.26	0%	n.s

(*) mean +/- relative standard deviation
(**) corrected for differences in baseline efflux between WT and ABCA1-/- cells

We noted that the fold increase in cholesterol efflux to apoA-I was consistently greater for wild-type astrocytes compared to microglia cultured from the same animals (2.5 fold vs. 1.2 fold, Figure 2-1, Table 2-1). Because our experiments were conducted in the absence of LXR/RXR stimulation in order to selectively examine the effect of ABCA1 on cholesterol efflux, it is possible that under these conditions astrocytes express all components of the pathway necessary for efficient cholesterol efflux to apoA-I, whereas additional factors may be required to elicit a strong efflux response to apoA-I in microglia.

Although surprisingly low efflux to exogenous apoA-I (1.2 fold) was observed in primary wild-type microglia, these cells did display significant cholesterol efflux to apoE2 (1.6 fold) and apoE3 (1.8 fold). Wild-type microglia showed poor efflux in the presence of apoE4 (1.5 fold), which did not reach statistical significance over baseline (Figure 2-1C, Table 2-1). Deficiency of ABCA1 in microglia blocked efflux to apoA-I and impaired cholesterol efflux by approximately 15% to apoE2 and apoE3 ($p<0.05$) compared to wild-type cells. Each apoE isoform was still able to elicit residual efflux activity between 1.3 – 1.6 fold in ABCA1-/- microglia (Figure 2-1C,

Table 2-1) suggesting that microglia also contain ABCA1-independent pathways to efflux lipid to apoE. For both wild-type and ABCA1-/- microglia, apoE3 tended to be the best acceptor, although no statistically significant difference was observed among any apoE isoform tested.

Taken together, these results suggest that ABCA1 facilitates cholesterol efflux to apoE, and is required for cholesterol efflux to apoA-I in astrocytes and microglia. Additionally, both astrocytes and microglia appear to contain ABCA1-independent pathways to efflux cholesterol to apoE. In astrocytes, apoE is as good a cholesterol acceptor as apoA-I, whereas apoE appears to be the preferred acceptor for microglia.

2.3.2 Lack of ABCA1 results in lipid accumulation in astrocytes and microglia

To determine if elimination of ABCA1-dependent efflux pathways results in cellular lipid accumulation in glia, we subjected wild-type and ABCA1-/- primary astrocytes and microglia to Oil-Red-O staining. Under normal culture conditions, lipids did not accumulate in efflux-competent wild-type astrocytes (Figure 2-2A), and only small lipid droplets were observed in wild-type microglia (Figure 2-2C). In contrast, ABCA1-/- astrocytes and microglia both accumulated numerous cytoplasmic lipid droplets, which were particularly abundant in microglia (Figure 2-2B, D). These observations suggest that ABCA1 is required to prevent excessive accumulation of neutral lipids in glial cells even in the absence of cholesterol loading.

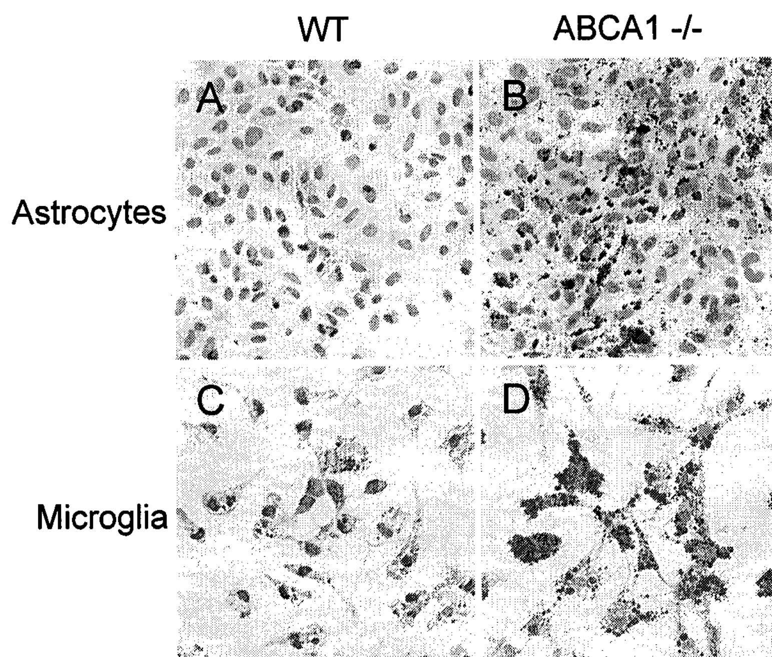


Figure 2-2: ABCA1-deficient glia accumulate lipids.

Primary wild-type (WT) and ABCA1-/- astrocytes (A,B) and microglia (C,D) were stained with Oil-Red-O to reveal accumulated neutral lipids, and nuclei were counterstained with hematoxylin (blue). Images were taken at 200X magnification. (A, B) Compared to wild-type astrocytes, ABCA1-/- astrocytes show increased intracellular levels of neutral lipids (red droplets). (C, D) Microglia, showing marked accumulation of lipid droplets in the absence of ABCA1 compared to wild-type cells.

2.3.3 ABCA1 facilitates apoE secretion from astrocytes and microglia

Antisense inhibition of ABCA1 expression has previously been reported to reduce apoE secretion from peripheral macrophages [63]. To assess the role of ABCA1 in apoE secretion from glial cells, we first determined the levels of intracellular apoE as well as the levels apoE secreted into conditioned medium. Initial experiments were conducted using medium conditioned for 72 h, where no differences in secreted apoE levels were observed between wild-type and ABCA1-/- glia (data not shown). Because both astrocytes and microglia express apoE receptors and are known to take up and resecrete apoE, we hypothesized that, at 72 h, apoE in the medium may have arisen from both newly synthesized apoE as well as re-secreted apoE. We therefore examined apoE secretion from both astrocytes and microglia after 6 and 8 h, respectively, in order to minimize the effect of resecreted apoE. For each well, intracellular

GAPDH levels were used to control for small variations in the total number of cells per well. Intracellular levels of apoE were equivalent in wild-type and ABCA1-/- astrocytes (Figure 2-3A,B, $p=0.72$). In contrast, ABCA1-/- conditioned media contained 50% less apoE than wild-type (Figure 2-3A,C, $p=0.0005$). As well, the proportion of total apoE released over 8 h into conditioned medium from primary wild-type and ABCA1-/- astrocytes was determined. This value was defined as apoE in the medium divided by the sum of intracellular and extracellular apoE, thus correcting for variations in intracellular apoE levels. The proportion of total apoE that was secreted into conditioned medium was reduced by 30% in ABCA1-/- compared to wild-type astrocytes (Figure 2-3D, $p<0.01$).

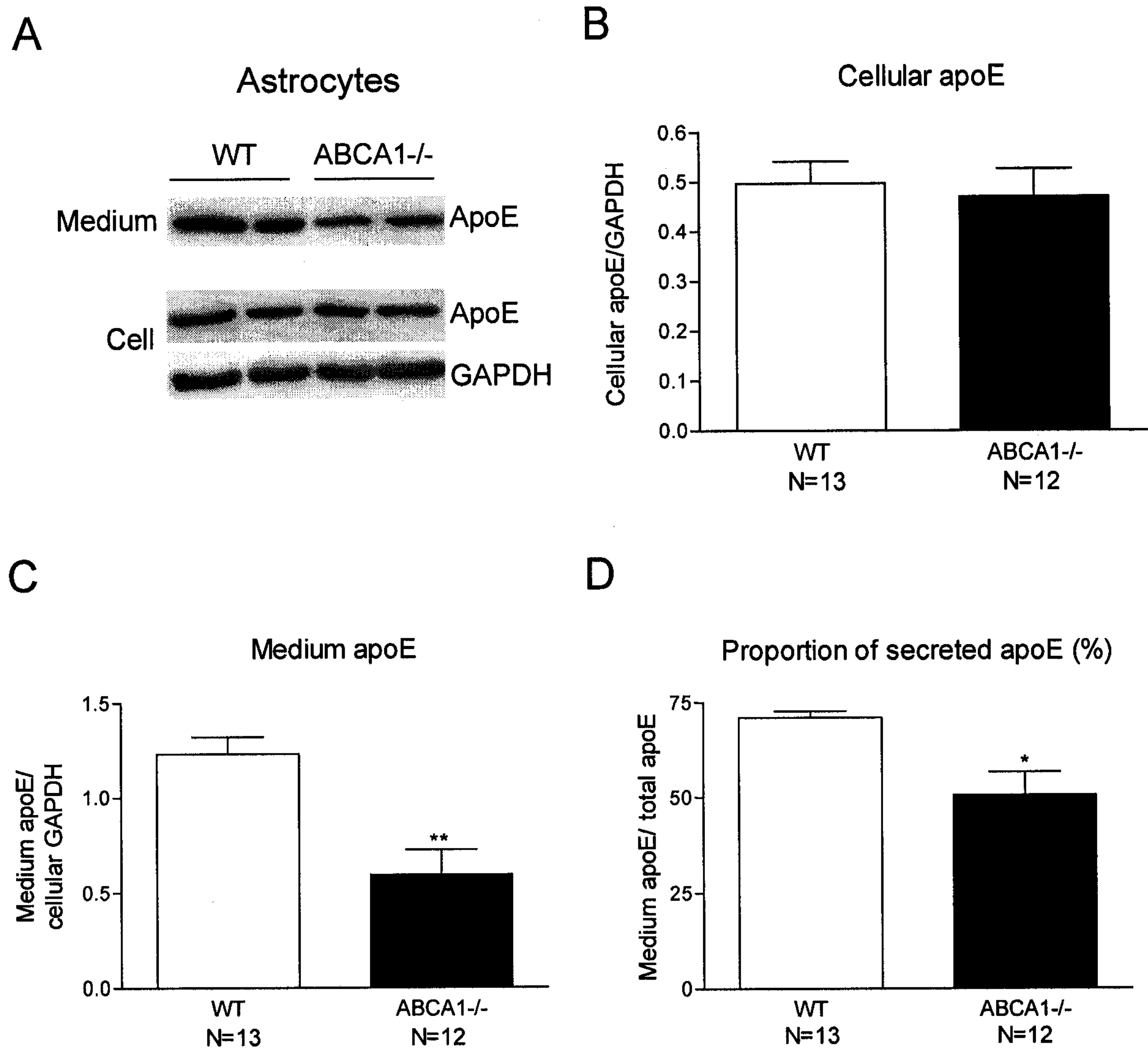


Figure 2-3: ABCA1 facilitates apoE secretion from astrocytes.

(A) ApoE levels in conditioned medium (upper panel) and whole cell lysates (middle panel) of wild-type and ABCA1-/- primary astrocytes were determined using a polyclonal antibody against murine apoE. Intracellular GAPDH (lower panel) was detected to control for equal protein loading and to normalize for small variations in cell number for measurement of secreted apoE. Bands were quantitated by densitometry. Graphs illustrate four independent experiments with N indicating the number of cultures prepared from individual mice. Data represent the mean and standard errors of apoE levels in whole cell lysates (B) and conditioned medium (C) of astrocytes, as well as the proportion of total apoE secreted into the medium (D). Student's t-test was used to determine significant differences in apoE levels. Welch's correction was applied where necessary. (* represents $p<0.01$ and ** represents $p<0.001$).

ABCA1 had a pronounced effect on apoE metabolism in microglia. Compared to wild-type microglia, deficiency of ABCA1 resulted in a 35% decrease in intracellular apoE levels (Figure 2-4A, B, $p=0.06$), and a 90% decrease in secreted apoE levels (Figure 2-4A, C, $p<0.05$), which

resulted in a 60% overall reduction in the proportion of total apoE secreted into conditioned media (Figure 2-4D, p<0.001).

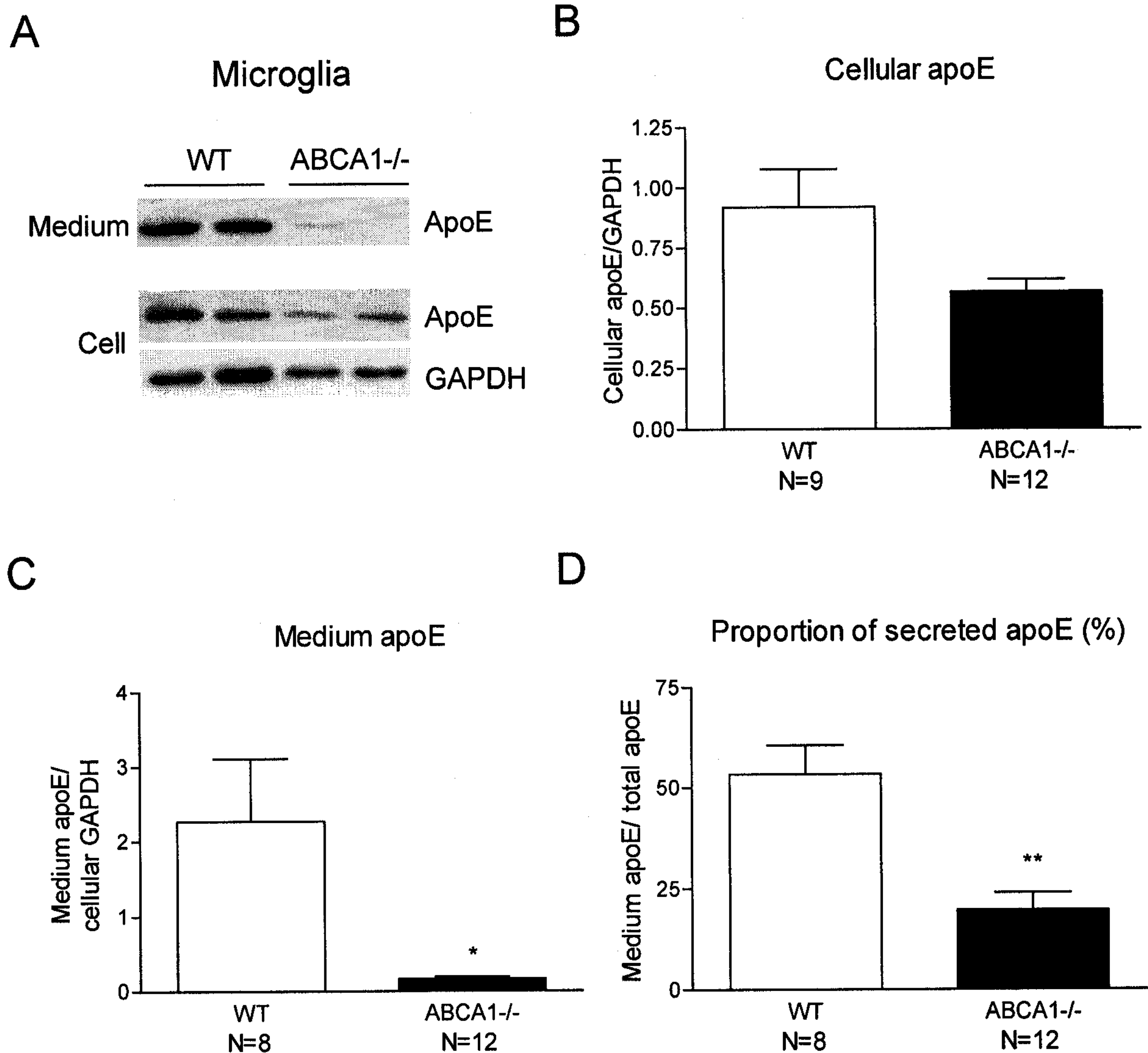


Figure 2-4: ABCA1-/- microglia are impaired in apoE secretion.

(A) ApoE levels in conditioned medium (upper panel) and whole cell lysates (middle panel) of wild-type and ABCA1-/- primary microglia were determined using a polyclonal antibody against murine apoE. Intracellular GAPDH (lower panel) was detected to control for equal protein loading and to normalize for variations in total cell number in measurements of secreted apoE. Bands were quantitated by densitometry. Graphs illustrate at least two independent experiments and N indicates the number of cultures prepared from individual mice. Data represent the mean and standard error of apoE levels in microglial whole cell lysates (B) and conditioned medium (C), as well as the proportion of total apoE secreted into the medium (D). Student's t-test was used to determine significant differences in apoE levels. Welch's correction was applied where necessary. (* represents p<0.05 and ** represents p<0.001).

2.3.4 ApoE secretion in wild-type microglia correlates with ABCA1 expression levels

Despite consistent culture conditions, we noted that wild-type microglia cultured from individual animals displayed a surprisingly large variability in apoE levels, particularly for apoE secreted into the medium. Because ABCA1 has previously been shown to affect apoE secretion from macrophages [63], we hypothesized that ABCA1 expression levels may influence the extent to which apoE is secreted in microglia, which are derived from the same lineage as peripheral macrophages. To assess this possibility, ABCA1 expression levels in microglia were determined by immunoblotting. Basal ABCA1 protein levels in wild-type microglia were found to be highly variable among equivalent amounts of wild-type microglia prepared from individual mice. Furthermore, ABCA1 levels correlated highly with apoE secretion (r^2=0.92, $p<0.0001$) (Figure 2-5A), as did apoE intracellular levels (r^2=0.82, $p<0.001$) (Figure 2-5B). In contrast to microglia, intracellular and medium apoE levels and ABCA1 expression levels were far more consistent among independent primary cultures of wild-type astrocytes. However, slight variations in levels of ABCA1 protein were again positively correlated with both medium (r^2=0.42, $p<0.005$) (Figure 2-5C) and cellular (r^2= 0.49, p=0.001) (Figure 2-5D) apoE levels. Taken together, these results provide further support for a role of ABCA1 in secretion of apoE, particularly in microglia. Finally, microglia showed a far greater range in ABCA1 and apoE levels in both media and cells compared to astrocytes (Figure 2-5).

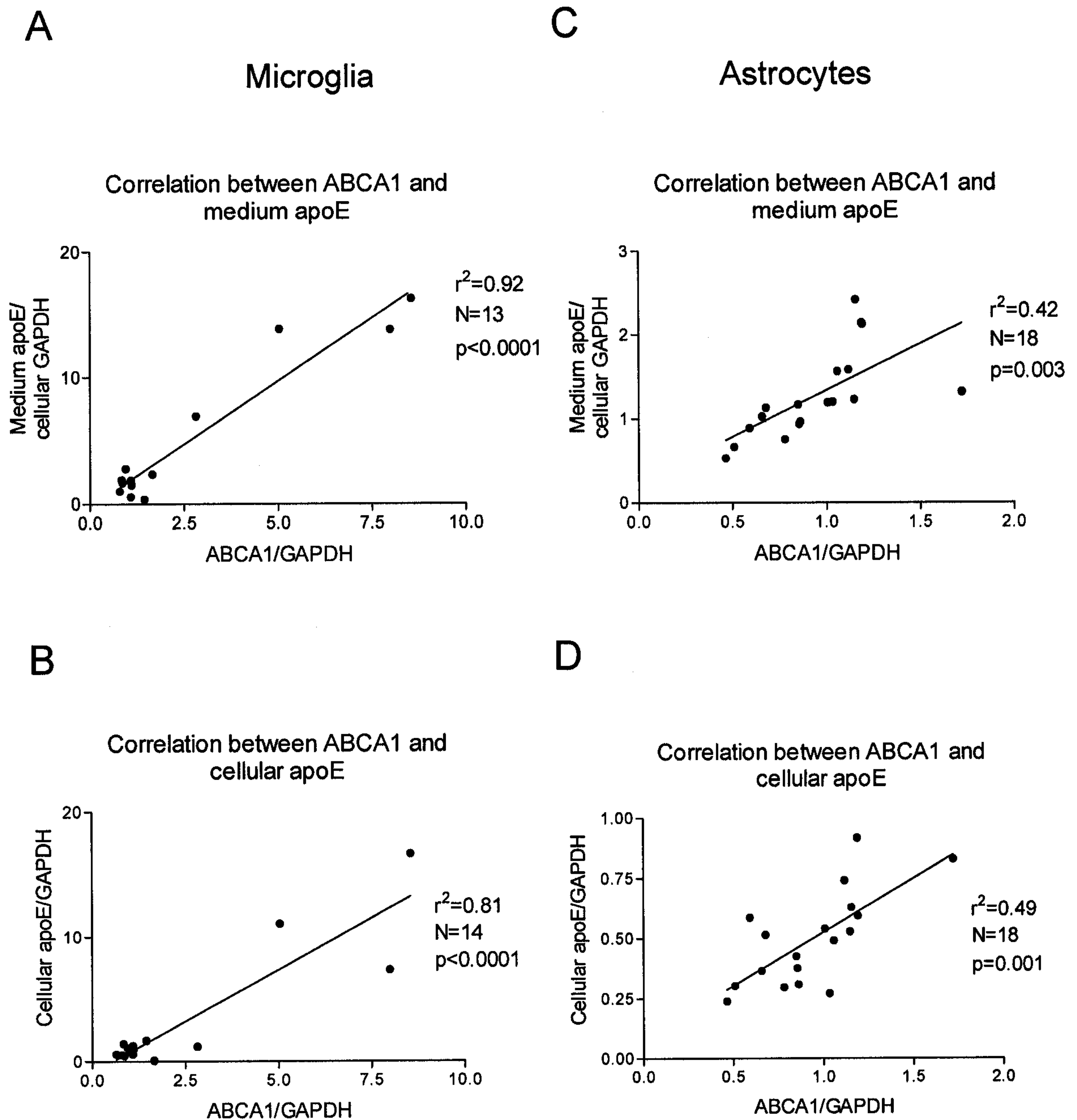


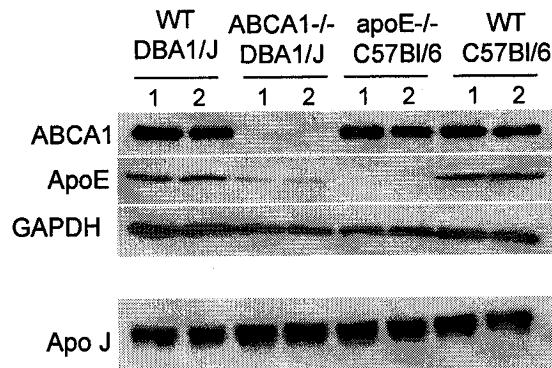
Figure 2-5: ABCA1 expression is correlated with both secreted and intracellular apoE levels in wild-type glia.

ABCA1 and apoE levels in wild-type microglia (A,B) and astrocytes (C,D) were normalized for intracellular GAPDH levels and correlated using linear regression analysis. Graphs of secreted (A,C) and intracellular (B,D) apoE represent pooled data of at least three independent experiments with N indicating the number of cultures prepared from individual mice.

2.3.5 ApoE levels are reduced in brain of ABCA1-deficient mice

The results presented above led us to hypothesize that ABCA1 may play a prominent role in the regulation of apoE homeostasis in murine brain. To assess the impact of ABCA1 deficiency on apoE levels *in vivo*, we first compared apoE protein levels in whole brain extracts prepared from age- and sex-matched ABCA1-/- mice and wild-type littermate controls on a DBA1/J genetic background. Because genetic strain has profound effects on murine lipids and lipoproteins [70, 71], whole brain extracts from age- and sex-matched apoE-deficient and wild-type controls from a pure C57Bl/6 genetic background were also prepared as additional controls. Western blot analysis showed that apoE levels in whole brains of male ABCA1-/- mice were reduced by approximately 65% compared to wild-type DBA1/J littermate controls ($p<0.01$) as well as age- and sex-matched wild-type C57Bl/6 mice ($p<0.01$) (Figure 2-6A, B). There were no significant differences in total brain apoE levels between wild-type DBA1/J and C57Bl/6 mice (Figure 2-6A, B). Identical results were obtained for mice evaluated at 2.5 months as well as at 6 months of age (data not shown). Compared to wild-type mice, ABCA1-/- mice also had 75% less apoE in plasma (data not shown), similar to the results reported by Wahrle *et al* [72].

A



B

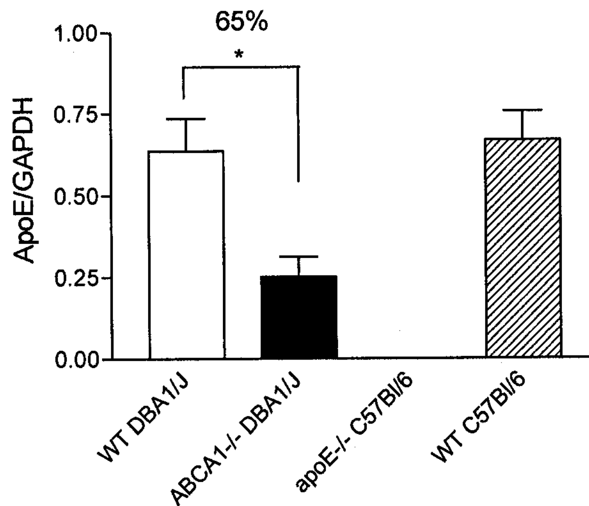


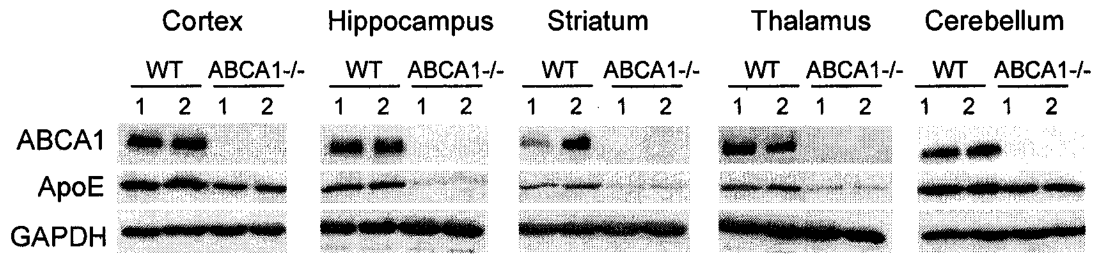
Figure 2-6: ApoE levels are reduced in brain of ABCA1-deficient mice.

(A,B) Male mice (WT DBA1/J, ABCA1-/- DBA1/J, apoE -/- C57Bl/6, or WT C57Bl/6) were sacrificed at 2.5 months of age. Gels are representative of total protein lysates prepared from whole brain of two individual mice per genotype. Tissue blots were immunodetected with ABCA1 (upper panel), apoE (second panel), GAPDH (third panel), and apoJ (fourth panel). GAPDH levels were used to control for equal protein loading. Bands were quantitated by densitometry, and graph represents pooled data from at least four mice per genotype, each measured in duplicate. One-way ANOVA with a Newman-Keuls post test was used to determine significant differences in brain apoE levels. (* represents $p<0.01$)

ApoJ is a second major brain lipoprotein that is synthesized and secreted from astrocytes and microglia. To assess whether loss of ABCA1 also affected the metabolism of apoJ, total brain extracts from wild-type and ABCA1-/- mice were immunoblotted for apoJ. In contrast to apoE, apoJ levels were indistinguishable among ABCA1-/- and wild-type DBA1/J littermate controls

as well as apoE-deficient and wild-type littermate C57Bl/6 controls (Figure 2-6A, lower panel), demonstrating that ABCA1 selectively influences apoE metabolism *in vivo*

To determine if ABCA1 affected apoE levels in a region-specific manner, individual brain regions from age- and sex-matched ABCA1-/- mice and wild-type littermate controls were then examined (Figure 2-7). Lack of ABCA1 affected apoE levels most severely in the hippocampus and striatum where apoE levels were reduced by 76% and 79% respectively (Figure 2-7A, B). ApoE levels were reduced by 61% in the thalamus and 41% in cortex (Figure 2-7A, B). In contrast, ABCA1 had the least impact in the cerebellum, where apoE levels were reduced by only 35% in ABCA1-/- compared to wild-type mice (Figure 2-7A, B).



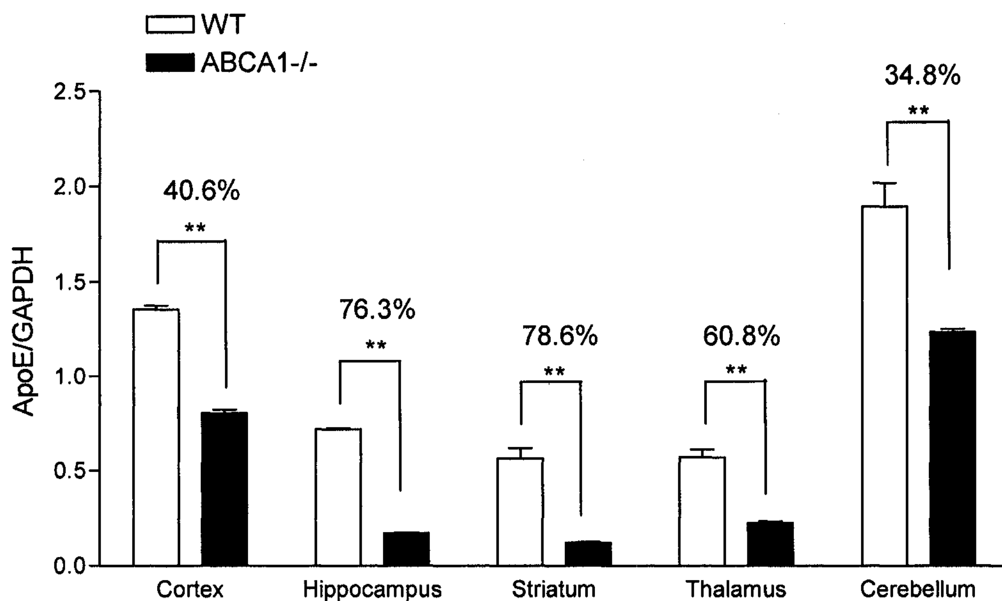


Figure 2-7: ABCA1-deficiency affects brain apoE levels in a region-specific manner.

(A) Male mice (WT DBA1/J and ABCA1-/- DBA1/J) were sacrificed at 3 months of age and individual brain regions were dissected. ABCA1 (upper panels) and ApoE (middle panels) were detected in each region by Western blot, quantitated by densitometry and normalized for GAPDH (lower panels), an internal protein loading control. (B) Graph represents mean and standard errors of two mice per genotype measured in duplicate. Student's t-test was used to determine significant differences in regional apoE levels. (** represents $p<0.001$)

Because the ABCA1-/- mice used in these studies lack circulating HDL and apoA-I due to loss of peripheral ABCA1 activity [73], it is formally possible that the changes in apoE levels observed in the brains of ABCA1-/- mice were simply due to loss of HDL or apoA-I, which is primarily

taken up in the brain from peripheral circulation [26]. To address this possibility, apoE levels were compared between total brain extracts prepared from age- and sex-matched wild-type and apoA-I-deficient mice, a model characterized by absent HDL and apoA-I. Deficiency of apoA-I had no impact on apoE levels in the brain (Figure 2-8), demonstrating that lack of circulating HDL and apoA-I in the ABCA1-/- mice is not sufficient to alter brain apoE levels *in vivo*.

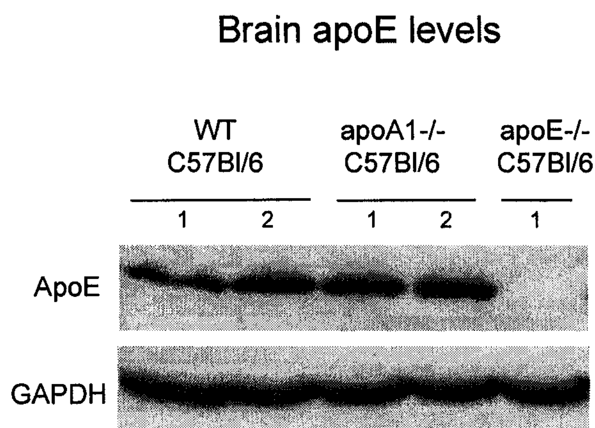


Figure 2-8: ApoE levels are not affected by apoA-I deficiency.

Total brain lysates of 2 month old male mice (WT C57Bl/6, ApoA1-/- C57Bl/6 or apoE -/- C57Bl/6) were immunodetected for apoE (upper panel) and GAPDH (lower panel) to control for equal protein loading. Western blot is representative of two mice per genotype and a single apoE-/- control.

2.4 Discussion

The identification of ABCA1 as an essential protein for the production of HDL has generated great interest in understanding ABCA1 function, particularly in tissues such as the liver and macrophages that are relevant to HDL metabolism and atherosclerosis. However, the nearly ubiquitous expression of ABCA1 suggests that it may also play roles in lipid transport in other cell types and tissues. Lipid metabolism in the CNS is largely independent of that outside the CNS, although there are several points of intersection that may be highly relevant to neurodegenerative diseases, particularly AD.

In this study, we provide evidence that lack of glial ABCA1 activity results in a 65% decrease in apoE levels *in vivo*, which is likely due to reduced apoE secretion from both astrocytes and microglia and a diminished pool of intracellular apoE in microglia. Additionally, we show that ABCA1 facilitates cholesterol efflux to exogenous apoE in primary astrocytes and microglia, as efflux is reduced in the absence of ABCA1 in both cell types. However, both astrocytes and microglia may also contain ABCA1-independent pathways to efflux cholesterol to apoE, as some residual efflux remains in ABCA1-/- cells. Our findings are in agreement with previous studies showing that macrophage ABCA1 facilitates cholesterol efflux to exogenous apoE [62, 74].

Under the experimental conditions used in our efflux studies, we did not observe marked apoE isoform specificity in a pattern that was consistent between astrocyte and microglial cultures. In astrocyte cultures, apoE2 was a slightly less potent acceptor than apoE3 or apoE4, but this trend was not observed in microglial cultures where no significant differences were observed in efflux among any of the apoE isoforms. Our findings are in contrast to a previous study suggesting that apoE2 elicits more cholesterol efflux than apoE3 or apoE4 [75]. The reasons why our findings differ from previous work are not clear, but may be due to subtle differences in culture preparation or efflux protocol. For instance, Michikawa *et al.* [75] used glia cultures derived from embryonic day 17-18 rat cerebral cortices, whereas we used postnatal day 1-2 murine cortices. In addition, each group used different procedures to separate astrocytes and microglia. More importantly, Michikawa *et al.* measured cholesterol efflux using [^{14}C]acetate as the source of labelled cholesterol, and cellular and media lipid levels were analyzed after a chloroform/methanol extraction procedure. In contrast, we used ^{3}H-cholesterol as efflux label, and measured radioactivity without separating organic and aqueous fractions.

In this study, we show that ABCA1 plays a critical role in the secretion of apoE from both astrocytes and microglia. These observations confirm and extend a previous study in which

ABCA1 was found to facilitate apoE secretion from human monocyte-derived macrophages [63]. We also demonstrate that ABCA1 is positively correlated with steady-state levels of intracellular apoE as well as with apoE secreted into the medium, both of which are measures of dynamic processes. Steady-state intracellular apoE levels reflect a balance between apoE synthesis and degradation of both newly synthesized and recycled apoE, whereas secreted apoE levels reflect a balance between apoE released from the cell coupled with reuptake of apoE by apoE receptors that are expressed in both astrocytes and microglia. Although the mechanisms by which ABCA1 affect apoE metabolism in glial cells are not yet understood, it is clear that lack of ABCA1 impairs the proportion of total apoE that is secreted into the medium in both astrocytes and microglia.

There are several potential mechanisms by which ABCA1 may affect apoE metabolism, which may or may not be linked functionally to lipid efflux. ABCA1 is known to cycle between the plasma membrane and late endosomal compartments [76] and, in addition to the plasma membrane, it has been suggested that ABCA1 may efflux cholesterol from late endosomes and lysosomes [67]. Moreover, apoA-I has been recently reported to be internalized and resecreted during ABCA1-mediated cholesterol efflux [77]. An increasing body of evidence indicates that internalized apoE does not undergo complete degradation and that it is resecreted [78-80]. For example, nascent lipoproteins recovered from livers of apoE-/- mice transplanted with wild type bone marrow, a model in which circulating apoE is derived exclusively from macrophages, showed that up to 60% of internalized apoE may be reutilized under physiological conditions [80]. Recently, HDL3 was shown to stimulate the recycling of internalized apoE and to act as an extracellular acceptor for recycled apoE in hepatoma cells [78]. In this study, apoE recycling was accompanied by cholesterol efflux and involved internalization of HDL3-derived apoA-I and its targeting to endosomes containing cholesterol and apoE [78]. Taken together, these observations suggest the possibility that apoE secretion and recycling may be coupled to cholesterol efflux and that ABCA1 may play a role in this process.

It is also possible to speculate ABCA1 could influence apoE secretion by affecting trafficking in secretory or recycling pathways. In this respect, plasma membrane-associated ABCA1 may have a distinct function compared to intracytoplasmic ABCA1. For example, plasma membrane-associated ABCA1 may function to transport cholesterol and phospholipids to various extracellular apolipoprotein acceptors, whereas intracytoplasmic ABCA1 may primarily participate in intracellular lipid trafficking. Additionally, it is known that apoE requires prenylation in order to be efficiently secreted [81], suggesting the possibility that any impact of ABCA1 on prenylation pathways might also affect apoE secretion. Although much remains to be learned, our results provide support for a role of ABCA1 in apoE secretion. Furthermore, Wahrle *et al* have reported that nascent particles released into conditioned medium from ABCA1-/- astrocytes contain less cholesterol than wild-type particles, suggesting that ABCA1 affects both secretion and lipidation of apoE in the CNS [72].

In vivo, total apoE levels are reduced by approximately 65% in whole brains of ABCA1-/- compared to wild-type animals, indicating a critical role for ABCA1 in maintaining normal apoE homeostasis in the CNS under physiological conditions. Loss of ABCA1 affects apoE levels most severely in the hippocampus and striatum, whereas cortex and thalamus are moderately affected by ABCA1 deficiency. ApoE levels in the cerebellum are minimally reduced in ABCA1-/- mice compared to wild-type controls. We hypothesize that defective secretion of apoE from astrocytes and microglia is a key factor that underlies the reduced apoE levels *in vivo*. In the absence of ABCA1, both astrocytes and microglia secrete less apoE, and the intracellular pool of microglial apoE available for secretion is also lower. Interestingly, elimination of ABCA1 had no effect on apoJ levels, suggesting that ABCA1 selectively regulates apoE metabolism rather than having a general effect on other brain-derived apolipoproteins. To our knowledge, whether deficiency of human ABCA1 results in changes in CNS or CSF levels of apoE has not been determined.

The *in vivo* reduction of brain apoE levels, coupled with the reduced ability of ABCA1-/- glia to maintain normal levels of cholesterol efflux and apoE secretion *in vitro*, suggests that ABCA1 deficiency may impair normal brain function, particularly in response to injury or disease. ApoE expression is known to be induced in glial cells in response to neuronal injury, where it is believed to participate in lipid transport processes involved in repairing neuronal membranes [36, 82]. Because ABCA1 deficiency results in region-specific reductions in apoE levels in brain, it is possible to speculate that ABCA1 may play a role in recovery from a variety of neurological insults by facilitating lipid mobilization via apoE.

Our observation that ABCA1-/- mice have a significant reduction in apoE levels in brain creates a paradox for predictions about the role of ABCA1 in AD. In this study, we provide the first direct evidence that glial ABCA1 affects apoE metabolism *in vivo* and *in vitro*. Reduced levels of apoE in ABCA1-/- brains may predict a greater susceptibility to neuronal injury due to decreased lipid-mediated repair pathways, yet lower levels of apoE may also delay amyloid deposition. In addition to glial ABCA1, neuronal ABCA1 may also participate in AD by affecting Aβ generation. At present, it is controversial whether ABCA1 increases or decreases Aβ generation, as two *in vitro* studies reported that increased ABCA1 expression reduced Aβ production [18, 83], whereas a third study reported the opposite findings [19]. Genetic variations in ABCA1 have been reported to alter the susceptibility to AD [84, 85], although additional studies are necessary to fully validate these results. Clearly, understanding the contributions of ABCA1 to AD will require *in vivo* studies in which the contribution of glial and neuronal ABCA1 to the pathogenesis of AD can be evaluated in an appropriate physiological context.

In summary, we demonstrate that glial ABCA1 is a key participant in apoE metabolism. We show that selective disruption of ABCA1 reduces cholesterol efflux to exogenous apoE and impairs apoE secretion from both astrocytes and microglia. As a result, ABCA1-/- glia

accumulate lipids under normal culture conditions. *In vivo*, ABCA1 is required to maintain normal levels of apoE in brain. Further, the effect of ABCA1 is specific to apoE and is independent of HDL and apoA-I levels. Taken together, our observations provide a clear and direct link between ABCA1 and apoE metabolism in brain.

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Chapter 3 - The absence of ABCA1 decreases soluble apoE levels but does not diminish amyloid deposition in two murine models of Alzheimer Disease*

3.1 Introduction

Alzheimer Disease (AD) is the most common cause of senile dementia and currently affects approximately 20% of the population over 80 years of age. Clinically, AD is characterized by severe impairments in memory and executive cortical functions as well as difficulties in language, calculation, visuospatial perception, behavior, and judgment [1]. Characteristic neuropathological hallmarks of AD include intraneuronal fibrillary tangles composed of hyperphosphorylated tau protein, and amyloid deposits that are composed largely of Aβ peptides, apolipoprotein E (apoE), lipids and other proteins that accumulate in the neural parenchyma and the cerebrovasculature [2, 3]. Aβ peptides are a heterogeneous group of peptides 39-43 amino acids in length that are proteolytically cleaved from amyloid precursor protein (APP) by β- and γ-secretases [4, 5]. Aβ40 and Aβ42 are the main Aβ species in the brain. Aβ42 is less soluble and present in all types of senile plaques, whereas Aβ40 is the major species deposited in cerebral blood vessels [4-7].

Most affected individuals have late-onset AD (LOAD) that typically manifests after 70 years of age. However, a number of families develop the disease in their 4th or 5th decades [8, 9]. The cases of familial AD (FAD) result from mutations within APP or secretase components [8, 10]. For

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example, the Swedish mutation (KM670/671NL) increases the amount of Aβ peptide that is generated from APP [11, 12]. Other APP mutations, including the Dutch (E693D) and Iowa (Q694N) mutations, alter the charge of the Aβ peptide and result in amyloid deposition predominately in the cerebral blood vessels rather than in the parenchyma [13-16]. In addition to mutations in APP, over 100 different mutations have been identified in presenilin-1 alone [17]. However, less than 5% of the overall clinical burden of AD is caused by mutations in APP and presenilins combined.

To date, the only well-established risk factor for LOAD is apolipoprotein E (apoE) [18, 19]. In the human population, apoE exists as three major alleles (apoE2, apoE3, and apoE4), and inheritance of an apoE4 allele increases the risk of developing AD at an earlier age, whereas inheritance of apoE2 delays the age of onset of AD [20-22]. How apoE functions in the pathogenesis of AD is a subject of intense investigation. ApoE is a component of several lipoprotein subclasses, including VLDL and HDL particles in the peripheral circulation, where apoE acts to mediate their uptake by apoE receptors [23, 24]. In the central nervous system (CNS), apoE is synthesized and secreted by astrocytes and microglia, and serves as the major cholesterol carrier in the brain and cerebrospinal fluid (CSF) [25-29]. In the brain, apoE expression is increased under conditions of chronic or acute neuronal damage, where it is believed to acquire lipids from damaged neuronal processes and redeliver these lipids to neurons during reinnervation [30-33].

In addition to lipid trafficking, apoE also plays a major role in Aβ metabolism. ApoE binds avidly to Aβ peptides and is found within amyloid plaques [34, 35]. ApoE is believed to play a major role in the conversion of Aβ peptides from soluble to fibrillar form, based on the observation that apoE-deficient animals are unable to form mature amyloid *in vivo* [36-40]. Although Aβ is deposited in the brains of apoE-deficient mice, the deposits do not contain

structural amyloid as defined by the ability to bind Thioflavine or Congo–Red dyes [36-40]. Furthermore, amyloid deposition is similar in distribution but less extensive in apoE heterozygous mice, suggesting a dose-dependent effect of apoE level on amyloid formation [41]. Therefore, factors that affect apoE abundance may also affect amyloid deposition and influence the onset or progression of AD.

The ATP-binding cassette transporter ABCA1 has recently been shown to modulate apoE levels in the brain, CSF, and plasma [42, 43]. In peripheral tissues, the primary biochemical function of ABCA1 is to transport cholesterol and phospholipids from the plasma membrane to lipid-free apoA-I, a reaction that constitutes the first step in high-density-lipoprotein (HDL) biogenesis [44]. The pre-β-HDL particles generated from this reaction circulate in the bloodstream and acquire additional lipids to form mature HDL particles. ABCA1-dependent lipid transport to apoA-I is the major pathway by which excess sterols from peripheral tissues are transported to the liver where they are converted into bile acids and excreted from the body. Mutation of one *ABCA1* allele causes familial hypoalphalipoproteinemia (FHA), a mild disorder of peripheral lipid metabolism characterized by reduced plasma HDL levels [45]. Mutation of both alleles of *ABCA1* results in Tangier Disease (TD), which is characterized by a nearly complete absence of plasma HDL, deposition of intracellular cholesterol esters, and, in some cases, an increased risk of cardiovascular disease [45-47].

In addition to high expression in liver and macrophages, ABCA1 is also abundant in the CNS [48-51]. ABCA1 is expressed in neurons, astrocytes, and microglia, and is transcriptionally induced by liver-X-receptor (LXR) and retinoic-X-receptor (RXR) agonists, similar to ABCA1 regulation in non-CNS cells [48-51]. Although the functions of ABCA1 in the brain are not completely understood, it is likely that ABCA1 participates in the regulation of lipid trafficking among the various cell types within the brain. We and others have shown that ABCA1 mediates cholesterol efflux to apoE and regulates the secretion of apoE from astrocytes and microglia [42,

[43]. Lack of ABCA1 results in accumulation of lipids in cultured glia, impaired secretion of glial-derived apoE, and a drastic reduction of apoE levels in the brain, particularly in the hippocampus and striatum [42, 43]. Furthermore, ABCA1-deficient glia exhibit impaired cholesterol efflux to apoE [42], and the low level of apoE that remain in the CSF and brains of ABCA1-deficient mice is poorly lipidated [43]. The observation that ABCA1 is a critical modulator of apoE abundance and lipidation in the CNS raises the possibility that ABCA1 may influence the onset or progression of AD through its effects on apoE.

Many previous studies have shown that intracellular cholesterol levels modify the cleavage of APP by secretases [52-57]. Low intracellular cholesterol favors the non-amyloidogenic cleavage of APP by α-secretase and leads to decreased Aβ generation, whereas high intracellular cholesterol favors APP cleavage by β- and γ- secretases, and thereby increases Aβ production [52-57]. *In vitro*, two recent studies showed that increased ABCA1 reduced the generation of Aβ peptides derived from human APP [51, 58], whereas a third study found that excess ABCA1 increased the generation of endogenous rodent Aβ [59]. The lack of consensus among these studies underscores the need to evaluate the role of ABCA1 on Aβ levels in a physiologically relevant *in vivo* setting.

Recently, Koldamova *et al* demonstrated that Aβ40 and Aβ42 levels were significantly reduced in APP23 mice treated with the LXR ligand TO901317, concomitant with increased ABCA1 expression in neurons [60]. Although these results support a role for neuronal ABCA1 in Aβ production *in vivo*, TO901317 induces the expression of several other genes in addition to *ABCA1*. For example, TO901317 has been reported to stimulate the expression of apoE in glial cell lines and in mice [61], although other groups have not observed significant increases in apoE expression in response to TO901317 [62, 63]. TO901317 also induces other members of the ABC-cassette transporter superfamily that may also participate in CNS lipid homeostasis.

Because ABCA1 is expressed in several cell types in the brain, ABCA1 may play multiple roles in the pathogenesis of AD. For example, neuronal ABCA1 may play a prominent role in regulating intraneuronal cholesterol content, thereby influencing the production of Aβ peptides. From this perspective, deficiency of ABCA1 would be predicted to increase intraneuronal lipids and lead to elevated Aβ levels. On the other hand, glial ABCA1 is crucial for normal apoE levels and lipidation [42, 43]. Because apoE is required for amyloid deposition [36-39], and deficiency of ABCA1 results in a 70% reduction in apoE levels, the absence of ABCA1 would be predicted to decrease amyloid deposition *in vivo*. Therefore, lack of ABCA1 could theoretically result in opposite outcomes in amyloid deposition depending upon whether the major role of ABCA1 is on Aβ production or Aβ clearance and deposition via apoE. Determining whether deficiency of ABCA1 increases, decreases, or has no net effect on Aβ and amyloid burden will therefore require investigating the effect of ABCA1 deficiency on Aβ and amyloid deposition *in vivo* where the physiological interactions among neurons and glia during the process of amyloid deposition are preserved.

We therefore crossed ABCA1-deficient mice to two independent murine models of AD to determine the impact of ABCA1 on amyloid burden, Aβ levels, and apoE levels *in vivo*. Here we report that elimination of ABCA1 has no measurable impact on the steady state levels of soluble or guanidine-extractable human Aβ when crossed to either model, and no effect on endogenous murine Aβ levels in the parental ABCA1-deficient mice. Lack of ABCA1 did result in the expected reduction in soluble apoE levels, consistent with previous results [42, 43]. Because apoE is required for amyloid deposition, we expected that each model would exhibit fewer amyloid plaques in the absence of ABCA1. However, despite a large reduction in apoE levels, amyloid burden was not diminished in either model. Our results support the hypothesis that ABCA1 has minimal impact on Aβ levels *in vivo*, but that ABCA1-mediated effects on

lipidation and levels of apoE may participate in the conversion of Aβ from soluble peptides to insoluble amyloid.

3.2 Experimental procedures

3.2.1 Mouse models

ABCA1-deficient mice were generously provided by Dr. Omar Francone (Pfizer Global Research and Development (Groton), and are on a DBA/1LacJ genetic background [64]. Tg-SwDI/B mice express the human APP770 cDNA containing the Swedish (KM670/671NL), Dutch (E693Q) and Iowa (D694N) mutations and are on a congenic C57Bl/6 genetic background [65]. Neuronal-specific expression is directed from the mouse Thy1.2 promoter. Beginning at 24-30 weeks of age, Thioflavine-S-positive amyloid deposits are observed primarily within the cerebrovasculature, as the Dutch and Iowa mutations alter the charge of the Aβ peptide and promote deposition in cerebral microvessels rather than in the neural parenchyma [65]. The APP/PS1 (line 85) mouse model (Jackson Laboratories) co-expresses two transgenes that are each expressed from the mouse prion promoter. One transgene is a chimeric mouse/human APP650 cDNA containing the Swedish (KM670/671NL) mutations, and the other is the human presenilin 1 (PS1) gene containing the DeltaE9 (deletion of exon 9) mutation [66]. Both transgenes are inserted at a single locus and are inherited together. The level of transgenic APP expression in the APP/PS1 mice is estimated to be approximately 2-4-fold over murine APP levels [66]. The APP/PS1 mice are maintained on a mixed F1 C3H/H3J x C57Bl/6 genetic background and develop parenchymal Thioflavine-S-positive amyloid deposits at approximately 36-40 weeks of age [66]. All animals were maintained on a standard chow diet (PMI LabDiet 5010, containing 24% protein, 5.1% fat, and 0.03% cholesterol). All procedures involving experimental animals were performed in accordance with protocols from the

Canadian Council of Animal Care and the University of British Columbia Committee on Animal Care.

3.2.2 Tissue collection

Mice were anesthetized with a mixture of 20 mg/kg Xylazine (Bayer) and 150 mg/kg Ketamine (Bimeda-MTC) and transcardially perfused with phosphate buffered saline (PBS) for 7 min. Brains were removed and divided into right and left hemispheres. Cortex and hippocampus were dissected from the right hemisphere and kept frozen at -80°C until analysis. The left hemisphere was immersion-fixed in 10% neutral buffered formalin for at least 48 h and cryoprotected in 30% sucrose in PBS at 4°C.

3.2.3 Protein extraction from brain tissue

Protein extractions from cortical and hippocampal regions were done in three consecutive steps in order to evaluate apoE, ABCA1, APP, and Aβ levels from the same animals. Firstly, brain regions were homogenized in approximately 8 volumes of ice-cold PBS containing Complete protease inhibitor (Roche) in a Tissuemite homogenizer. The homogenate was centrifuged at 4°C for 45 min at 12,500 rpm in a microfuge (Eppendorf). The supernatant (soluble fraction) was removed and used to evaluate soluble apoE. No ABCA1 or APP is detectable in this fraction, as both are membrane-bound proteins. The pellets from the PBS solubilization step were then resuspended in ice-cold lysis buffer (containing 10% glycerol, 1% TritonX-100 and Complete protease inhibitor (Roche) in PBS) and centrifuged at 4°C for 10 min at 9,000 rpm in order to extract ABCA1 and APP. The pellet from this step (insoluble fraction) was finally solubilized in 5 M guanidine hydrochloride in 50 mM Tris–HCl, pH 8.0 at room temperature for about 2.5–3 h with continuous rotation in order to evaluate plaque-associated Aβ and apoE. Brain tissue from all animals was extracted in the identical manner, and all

fractions were immediately frozen at -80°C until analysis. Protein concentrations were determined by DC Protein Assay (BioRad).

3.2.4 Western blotting

ApoE, ABCA1 and APP levels were determined by Western blot. For soluble apoE (PBS fraction), and ABCA1 and APP (lysis buffer fractions), protein concentrations were determined by DC Protein assay (BioRad) prior to analysis and equal amounts of protein were electrophoresed through 10% SDS polyacrylamide gels, electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane (Millipore) and immunodetected using a murine-specific apoE antibody (Santa Cruz), a monoclonal anti-ABCA1 antibody raised against the second nucleotide binding domain (NBD2 of ABCA1) 48, 22C11 which detects both murine and human APP (Chemicon), or an anti-GAPDH antibody (Chemicon) as a loading control. Blots were developed using enhanced chemiluminescence (Amersham) according to the manufacturer's recommendations. For insoluble apoE, protein loading was normalized by Coomasie blue staining of duplicate gels. Bands were quantified by densitometry using NIH Image J software.

3.2.5 Aβ measurements

Human Aβ levels were quantified by ELISA (Biosource) according to the manufacturer's protocol. Samples were analyzed diluted in reaction buffer as described in the manufacturer's protocol. Endogenous murine Aβ levels were quantified using 75 μg of protein from total hippocampal homogenates. The coating antibody used was R163 [67, 68] 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% bovine serum albumin for 2h at room temperature (RT). Following 5 washes with TBS-T (Tris-buffered saline

containing 0.1% Tween-20), samples were incubated for 2h at RT with biotinylated 4G8 (Signet Laboratories Inc., Dedham, MA) for 1h at RT with agitation. The plates were washed 5X and streptavidin-alkaline phosphatase complex was added for 1h at RT. Plates were finally washed 5X with TBS-T and once with water and AttoPhos reagent (Calbiochem, San Diego, CA) was added for 30-60 min and a reading was taken on a Bio-Tek FL600 fluorescence microplate reader. Human and murine Aβ levels were normalized to total protein as measured by DC Protein Assay (BioRad).

3.2.6 Histological analysis

25μm-thick, coronal sections were cut on fixed brains using a cryostat from the genu of the corpus callosum to the most caudal hippocampus. For Thioflavine-S staining, sections were immersed for 10 min in 1% Thioflavine-S solution followed by washing and dehydration in increasing ethanol concentrations from 70%-100% followed by xylene. Slides were mounted in DPX mounting medium (DBH) and imaged within the following 24 h. Aβ immunohistochemistry was performed as previously reported [69] with slight modifications. Briefly, floating sections were incubated in 88% formic acid for 5 min, incubated for 30 min with 0.3% H_2O_2 in PBS-T (0.01 M phosphate buffered saline, pH 7.4, containing 0.3% Triton X-100), transferred into 1% horse serum (Vector Laboratories) in PBS-T for 30 min and incubated overnight at 4°C in 1.5% horse serum in PBS-T containing antibodies 4G8 and 6E10 (Chemicon) (1:1000 dilution each) against residues 17-24 and 1-17 of Aβ, respectively. Sections were then washed with PBS-T, treated for 1 h at room temperature with a biotinylated anti-mouse antibody (1:1000, Vector) followed by incubation in avidin-biotinylated horseradish peroxidase complex (ABC Elite, Vector Laboratories; 1:1000) for 1 h at room temperature. Peroxidase labeling was visualized by incubation in 0.05% 3,3-diaminobenzidine (DAB, Sigma) and 0.001% H_2O_2 in 0.05 M Tris–HCl buffer, pH 7.6. After a 2 minute incubation period, sections were washed, mounted on glass

slides, and coverslipped with DPX mounting medium. Imaging was performed on a Zeiss Axioplan 2 microscope using Metamorph capture software.

3.2.7 Quantification of amyloid and Aβ burden

Amyloid load was quantified in different brain regions depending on the mouse model. For the hippocampus (Tg-SwDI/B and APP/PS1), six sections, 300 µm apart and spanning the entire length of the hippocampus were chosen for staining and subsequent analysis. For the thalamus (Tg-SwDI/B), four sections, 300 µm apart, starting at the most rostral aspect of the thalamus were chosen. Quantification of amyloid in the cingulate (Cg) cortex (APP/PS1) was performed on sections before the emergence of the genu of the corpus callosum on a total of four sections, 300 µm apart. This systematic sampling strategy was chosen to account for subregional variations of hippocampal size and potential subregional variations in plaque burden. The slides were viewed in a Zeiss Axioplan 2 microscope under light of wavelength 450-490 nm. Colored images were captured using Metamorph software and a Cool Snap HQ camera (Photometrics). Camera settings were adjusted at the start of the experiment and maintained for uniformity. Using Metamorph software, low magnification (2.5x) images were acquired. A computer generated grid composed of 16 higher magnification (10x) fields was superimposed on each 2.5x image. Throughout the hippocampus, 50% of the 10x fields were randomly selected and images of these fields were acquired for analysis. Areas of the regions of interest in each 10x image were outlined manually. The plaque/amyloid area within the fields of interest was identified by color and intensity-level threshold, the level of which was maintained throughout the experiment. After manual editing of staining artifacts, such as undissolved Thioflavin-S particles, the thresholded and total areas, and the amyloid load defined as (sum of Thioflavine-S staining area measured/sum of field area analyzed) x 100 was calculated for each mouse. Images were quantified by observers blinded to genotype.

For Aβ load, stained sections were chosen using the same sampling method using sections cut immediately before or after those stained with Thioflavine-S. Grayscale images were captured and analyzed as above. The Aβ load was defined as the sum of Aβ immunoreactive area measured/sum of field area analyzed) x 100 and was determined by observers blinded to genotype.

The number of Thioflavine-S positive plaques per unit area was also calculated for hippocampus and a larger cortical area in the APP/PS1 mice. For this procedure, plaques visible on 2.5x images were counted manually by observers blinded to genotype. The boundaries of the hippocampus were used, while limits of the larger cortical area were determined by transecting each image with a horizontal line through the corpus callosum and counting all Thioflavine-S positive plaques above this line. Plaque count totals were averaged between two independent observers blinded for genotype, corrected for total area and expressed as the number of Thioflavine-S positive plaques/μm^2.

3.2.8 Statistical analysis

Data is shown as mean±standard error. One-way ANOVA with a Newman-Keuls post test or two-tailed unpaired Student's t-tests were used for statistical analysis. In the t-test analyses, Welch's correction for unequal variances was applied when variances were significantly different between groups. All statistical analyses were performed using Graphpad Prism (version 4.0; Graphpad Software for Science Inc., San Diego, CA).

3.3 Results

3.3.1 ABCA1 levels are unaffected by the APP and PS1 transgenes

To evaluate whether the presence of an APP or PS1 transgene affected ABCA1 expression, we first determined ABCA1 protein levels in the hippocampus and cortex in two mouse models of AD. Tg-SwDI/B mice express human APP containing the Dutch (E693Q) and Iowa (D694N) mutations in neurons under the control of the mouse Thy1 promoter 65. APP/PS1 mice use the murine prion promoter to co-express a chimeric mouse/human APP650 cDNA containing the Swedish (KM670/671NL) mutations, as well as the human presenilin 1 (PS1) gene containing the DeltaE9 mutation 66. Tg-SwDI/B animals were examined at 10 months of age, and contained abundant ABCA1 protein in wild-type mice and no detectable ABCA1 in ABCA1-/- mice (Figure 3-1A). No effect of the APP transgene on ABCA1 levels was observed (Figure 3-1A). APP/PS1 mice were examined at 12 months of age. As expected, ABCA1 levels were undetectable in the hippocampus of ABCA1-/- mice, and were reduced by approximately 60% in ABCA1+/- mice compared to wild-type controls irrespective of the presence of the APP or PS1 transgenes (Figure 3-1B). Similar results were observed in cortex of APP/PS1 mice (data not shown).

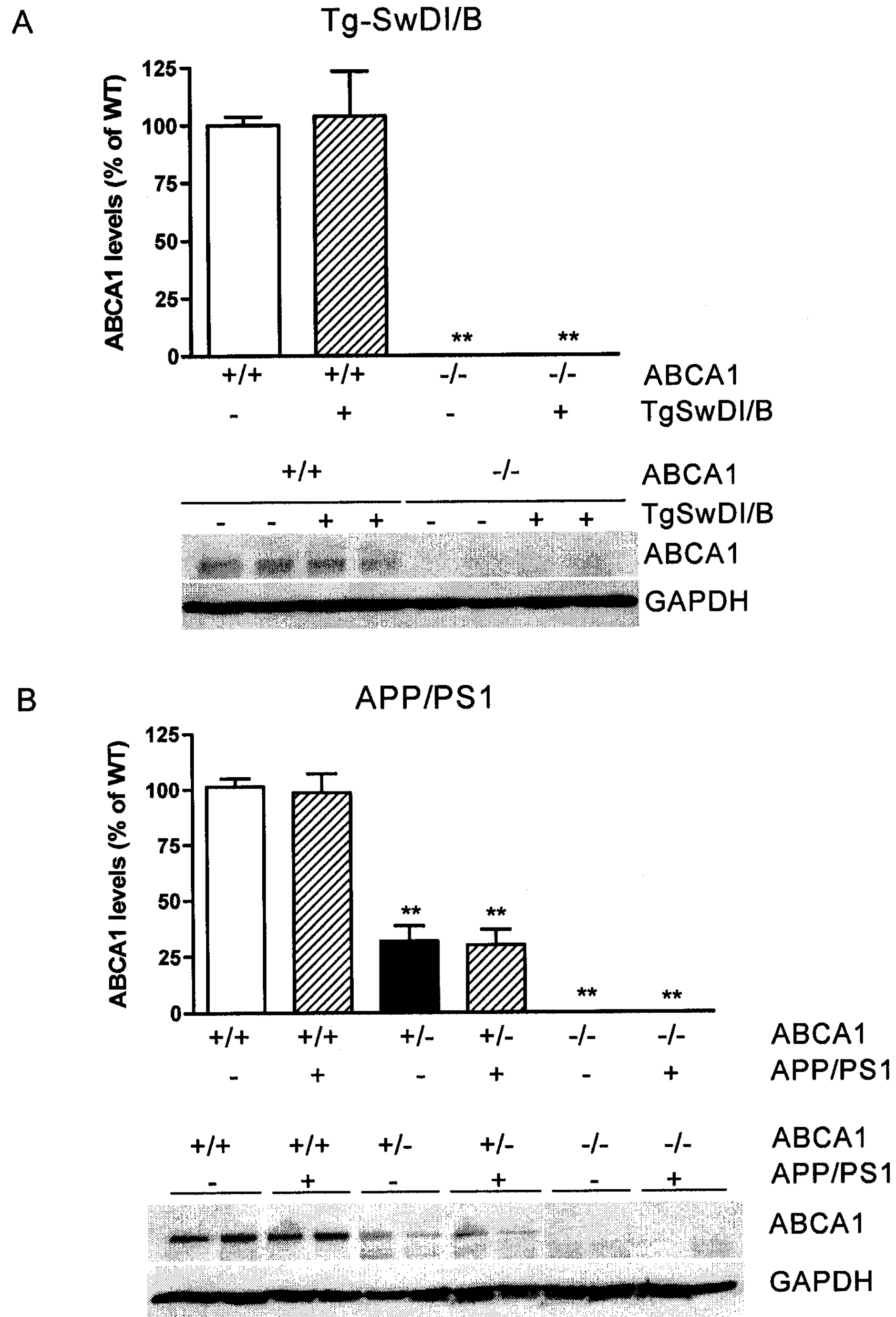

Figure 3-1: Presence of the APP transgene does not modify ABCA1 abundance

ABCA1 protein levels in the hippocampus of Tg-SwDI/B (A) and APP/PS1 (B) mice were determined by Western blot and quantitated by densitometry. Graphs are expressed as % of wild-type (APP-, ABCA1+/+ mice were assigned a 100%) and illustrate at least two independent experiments. (A) Data corresponds to four individual mice per genotype [2 female (F), 2 male (M)]. (B) Data represents APP-, ABCA1+/+ (n=5 [3F, 2M]), APP+, ABCA1+/+ (n=5 [2F, 3M]), APP-, ABCA1+/- (n=6 [3F, 3M]), APP+, ABCA1+/- (n=6 2F, 4M]), APP-, ABCA1-/- (n=5 [2F, 3M]) and APP+, ABCA1-/- (n=5 [2F, 3M]). Western blots show two representative samples per group. GAPDH was used as an internal loading control. ** represents $p<0.001$ compared to wild-type (APP-ABCA1+/+) by ANOVA with Newman-Keuls post test.

3.3.2 Deficiency of ABCA1 does not affect APP or Aβ levels *in vivo*

Western blots of brain lysates were then used to determine whether deficiency of ABCA1 affected APP expression *in vivo*. As expected, total APP levels were significantly higher in lysates of transgenic Tg-SwDI/B (Figure 3-2A) and APP/PS1 (Figure 3-2B) brains compared to non-transgenic controls. APP protein levels were identical in wild-type, heterozygous, and ABCA1-deficient mice in both Tg-SwDI/B and APP/PS1 models (Figure 3-2A, B), demonstrating that ABCA1 does not influence APP abundance. In contrast to the reported 50% increase in APP levels over endogenous levels in the Tg-SwDI/B model 65, we observed that transgenic mice expressed 2-3 fold more APP than controls in our cohorts. Possible explanations for this discrepancy include different protein extraction protocols and the different genetic background of the animals upon breeding to ABCA1 hemizygous mice.

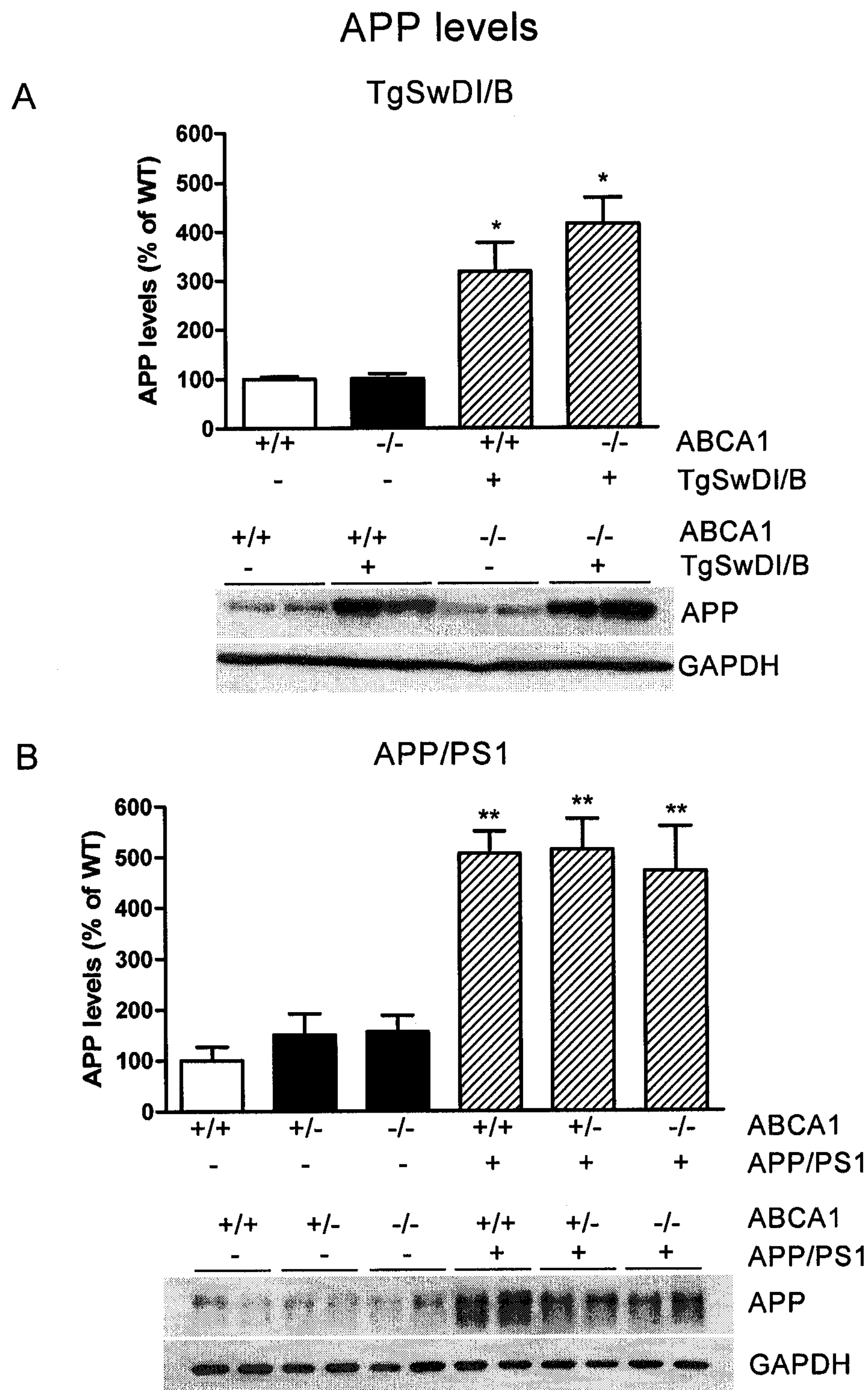

Figure 3-2: APP levels are unaffected in the absence of ABCA1.

APP protein levels in hippocampus of Tg-SwDI/B mice (A) and cortex of APP/PS1 mice (B) were determined by Western blot and quantified by densitometry. Graphs are expressed as % of wild-type (APP-, ABCA1+/+ mice were assigned a 100%) and illustrate at least two independent experiments. (A) Data corresponds to four individual mice per genotype [2F, 2M]. (B) Data represents four individual mice per genotype [2F, 2M]. Western blots show two representative samples per group. GAPDH was used as an internal loading control. * represents $p<0.01$ and ** represents $p<0.001$ compared to wild-type (APP-, ABCA1+/+) by ANOVA with Newman-Keuls post test.

Because ABCA1 has previously been reported to either increase [59] or decrease [51, 58] Aβ production in cultured cells, we next evaluated whether ABCA1 affected the steady state levels of human Aβ in brains of Tg-SwDI/B and APP/PS1 mice with various *ABCA1* gene doses. Several serial extraction protocols have been developed to remove different Aβ pools from brain tissue. We developed an extraction protocol to allow the levels of Aβ to be determined from the same brain samples that were used to measure ABCA1, APP and apoE protein levels. Brains were first extracted in PBS and then in lysis buffer, and soluble and weakly-membrane-associated Aβ was measured from each fraction. No significant differences in Aβ40 levels were observed in either fraction from either model (Figure 3-3A-D). PBS-soluble and lysis-buffer soluble Aβ42 levels were below the detection limit of our assay (data not shown). We also evaluated the impact of ABCA1 deficiency on steady-state levels of endogenous, murine Aβ40 in the parental ABCA1-deficient mice, and found that murine Aβ40 levels were indistinguishable among wild-type, heterozygous, and ABCA1-deficient mice in cortex and hippocampus (Figure 3-3E, F). These results demonstrate that the abundance of soluble Aβ species is not elevated in the absence of ABCA1.

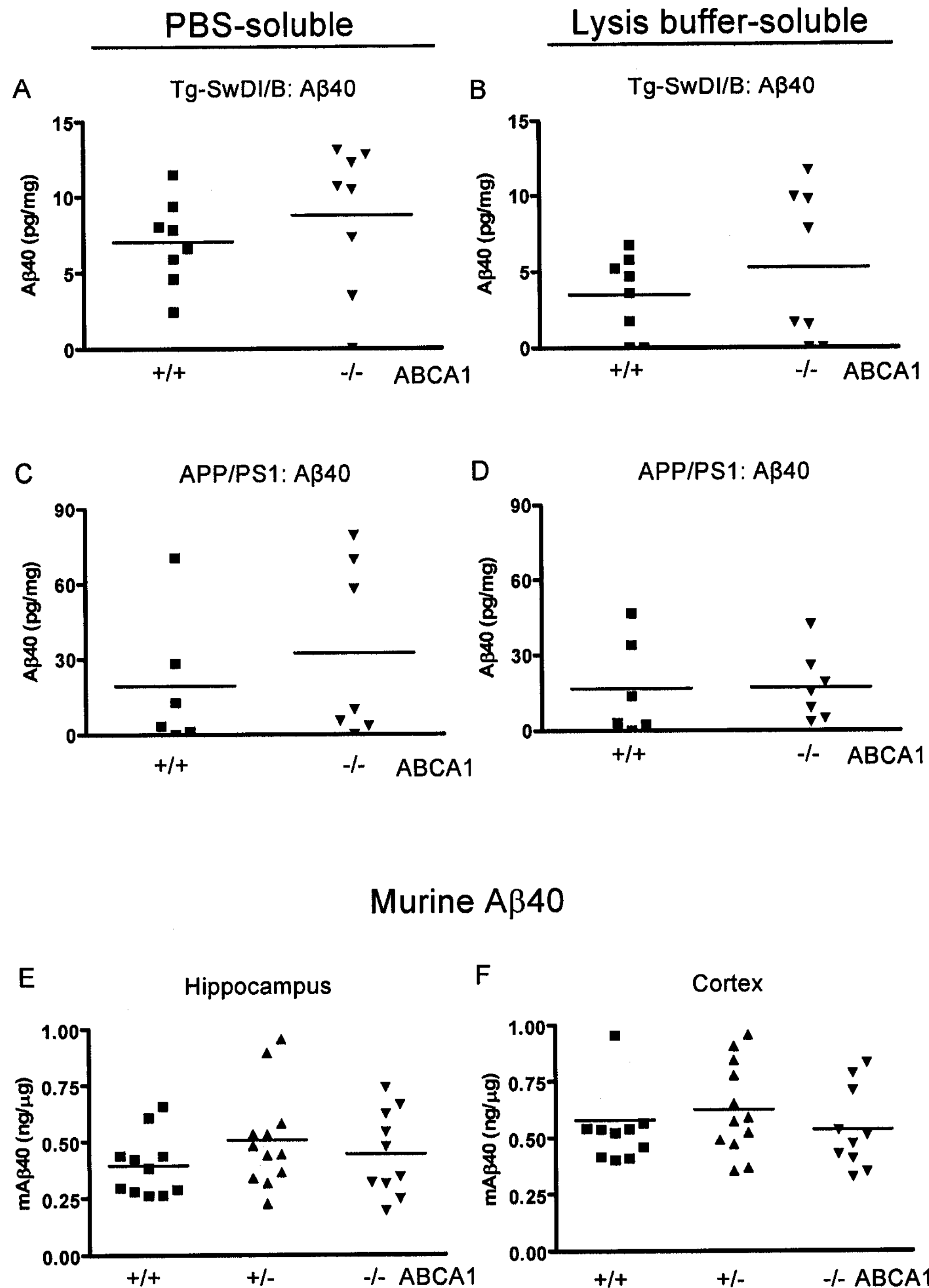

Figure 3-3: Soluble Aβ levels are not affected by lack of ABCA1.

Soluble Aβ40 levels in Tg-SwDI/B hippocampi were quantified from PBS (A) and lysis buffer (B) extracts. Soluble Aβ40 levels in APP/PS1 cortex were quantified from the PBS (C) and lysis buffer (D) extracts. ELISA values were normalized to total protein as determined by DC Protein Assay. Graphs correspond to Tg-SwDI/B+, ABCA1+/+ (n=8 [4F, 4M]) and Tg-SwDI/B+, ABCA1-/- (n=8 [4F, 4M]) (A and B), and to APP+, ABCA1+/+ (n=6 [2F, 4M]), APP+, ABCA1-/- (n=7 [3F, 4M]) (C and D). Endogenous murine Aβ40 levels in hippocampus (E) and cortex (F) were determined by ELISA in ABCA1+/+ (n=11 [6F, 5M]), ABCA1+/- (n=12 [6F, 6M]) and ABCA1-/- (n=10 [6F, 4M]) mice. Values were normalized to total protein.

To facilitate comparison of insoluble Aβ levels in our studies to several other relevant publications [39, 70, 71], we elected to use 5 M guanidine to extract Aβ that is likely to be strongly associated with membranes or deposited within plaques. No significant differences were observed in steady-state guanidine-extractable Aβ40 or Aβ42 levels in either model (Figure 3-4). These data are summarized in Table 3-1, and suggest that deletion of ABCA1 has no significant impact on either transgenic or endogenous steady state Aβ levels *in vivo*.

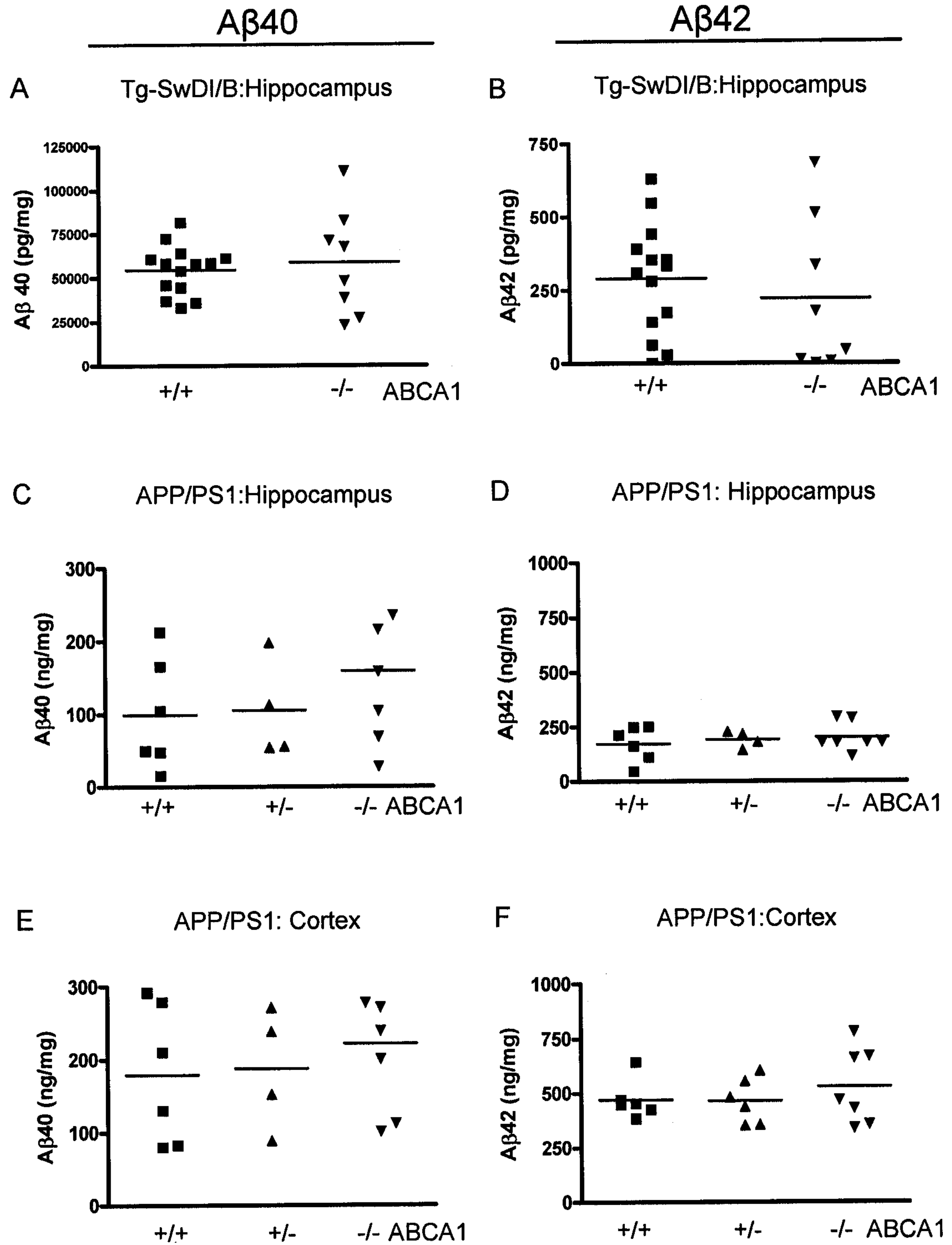

Figure 3-4: Guanidine-extractable Aβ levels are independent of ABCA1 genotype.

Aβ40 and Aβ42 levels in hippocampus of Tg-SwDI/B (A and B respectively), and in hippocampus (C and D) and cortex (E and F) of APP/PS1 mice were measured by ELISA. Values were normalized to total protein as determined by DC Protein Assay. Graphs correspond to Tg-SwDI/B+, ABCA1+/+ (n=14 [8F, 6M]) and Tg-SwDI/B+, ABCA1-/- (n=8 [4F, 4M]) (A and B), and to APP+, ABCA1+/+ (n=6 [2F, 4M]), APP+, ABCA1+/- (n=at least 4 [2F, 2M]) and APP+, ABCA1-/- (n=7 [3F, 4M]) (C to F).

Table 3-1: Aβ levels, Aβ immunoreactivity and amyloid load by region and mouse model in the presence or absence of ABCA1

Hippocampus

		wild-type			ABCA1-/-			
		mean	SD	n	mean	SD	n	p value
TgSwDI/B	Aβ40 (pg/mg)	54626	13991	14	58701	29958	8	n.s.
	Aβ42 (pg/mg)	288.9	189	14	221.9	262.8	8	n.s.
	Aβ load (%)	0.1	0.063	8	0.26	0.169	8	<0.05
	Amyloid load (%)	0.076	0.058	8	0.255	0.125	8	<0.005
APP/PS1	Aβ40 (ng/mg)	99.25	76.74	6	159.3	99.11	7	n.s.
	Aβ42 (ng/mg)	172.7	82.4	6	203	65.95	7	n.s.
	Aβ load (%)	1.56	0.97	4	0.79	0.38	5	n.s.
	Amyloid load (%)	0.62	0.35	6	0.73	0.44	7	n.s.
	Plaque # (plaques/μm^2)	1.71e-05	1.00e-05	6	2.54e-05	1.33e-05	7	n.s.
Parental ABCA1-/-	Aβ40 (ng/μg)	0.394	0.137	11	0.445	0.189	10	n.s.

Cortex

		wild-type			ABCA1-/-			
		mean	SD	n	mean	SD	n	p value
TgSwDI/B	Aβ40 (pg/mg)	ND			ND			
	Aβ42 (pg/mg)	ND			ND			
	Aβ load (%)	ND			ND			
	Amyloid load (%)	ND			ND			
APP/PS1	Aβ40 (ng/mg)	179.2	94.87	6	222.5	91.66	7	n.s.
	Aβ42 (ng/mg)	470.2	89.6	6	529.7	172.8	7	n.s.
	Aβ load (%)	ND			ND			
	Amyloid load (%)	1.16	0.55	6	0.99	0.26	7	n.s.
	Plaque # (plaques/μm^2)	4.21e-05	1.65e-05	6	4.74e-05	5.74e-06	7	n.s.
Parental ABCA1-/-	Aβ40 (ng/μg)	0.577	0.208	11	0.535	0.179	10	n.s.

ND: not determined

3.3.3 ABCA1 deficiency increases Aβ deposition in Tg-SwDI/B mice

To evaluate the pattern and quantity of Aβ deposits, hemicoronal sections of Tg-SwDI/B and APP/PS1 brains were stained for Aβ deposits as described. In 10 month-old Tg-SwDI/B mice, there was a significant 2-fold increase in Aβ immunoreactivity in the absence of ABCA1 compared to controls, (Table 3-1), and the staining distribution pattern was indistinguishable between groups (Figure 3-5). Most Aβ immunoreactivity in the hippocampus was observed in the subiculum and the polymorph layer of the dentate gyrus (Figure 3-5A). In 12- month old APP/PS1 mice, no significant differences in the distribution pattern or quantity of Aβ deposits were noted in the hippocampus of ABCA1-deficient animals compared to wild-type littermate controls (Figure 3-5B, Table 3-1). Deposits were observed throughout the hippocampus and cortex of APP/PS1 animals.

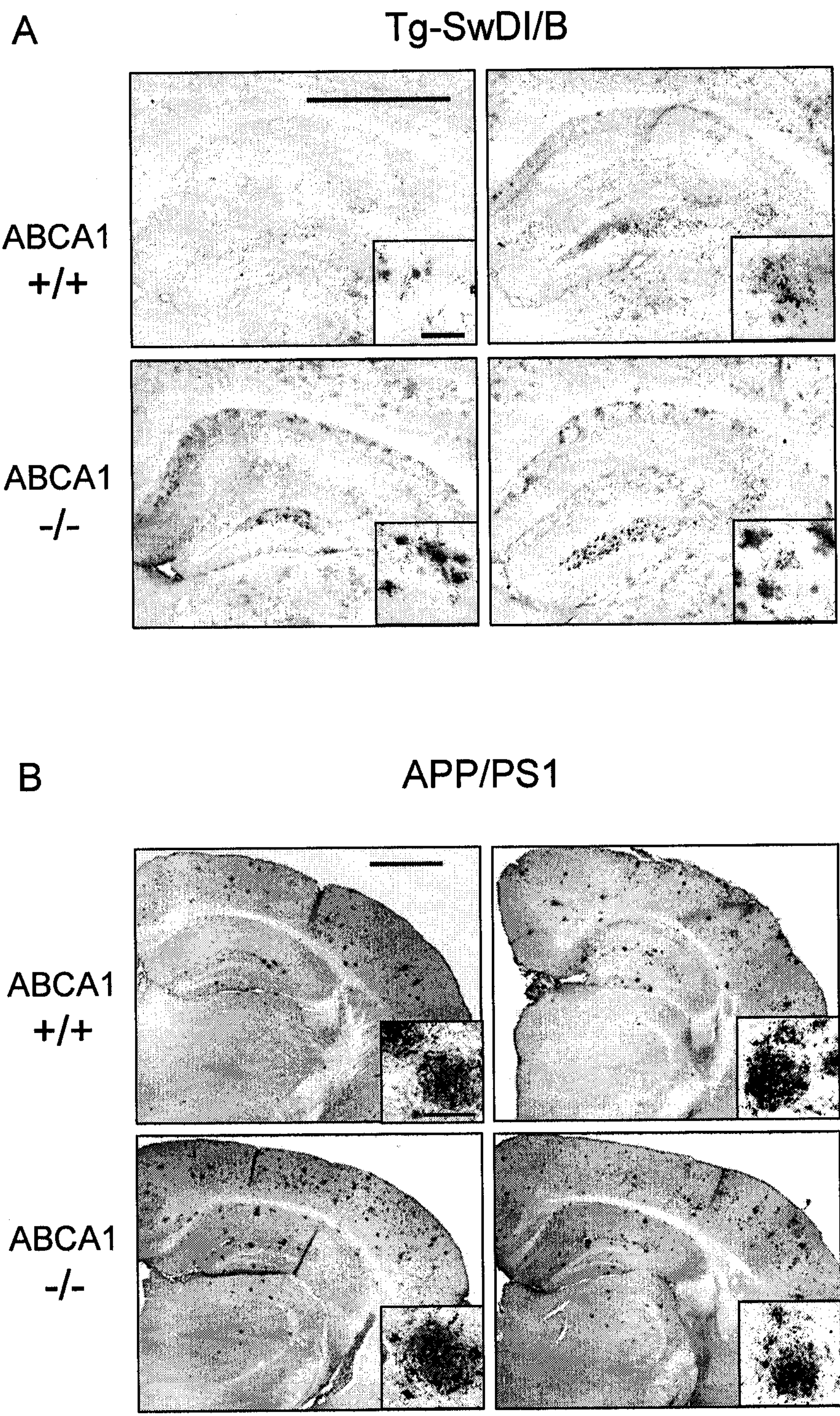

Figure 3-5: Absence of ABCA1 does not alter Aβ distribution and elevates Aβ load in Tg-SwDI/B mice.

Immunohistochemistry for Aβ was performed as described in Tg-SwDI/B+, ABCA1+/+ (n=8 [4F, 4M]) and Tg-SwDI/B+, ABCA1-/- (n=8 [4F, 4M]) (A) and in APP+, ABCA1+/+ (n=4 [1F, 3M]), APP+, ABCA1+/- (n=5 [2F, 3M]) and APP+, ABCA1-/- (n=5 [2F, 3M]) (B) brains. Pictures correspond to two representative mice per genotype at 2.5x magnification. Insets correspond to 40x magnification of individual plaques. In panel (A), scale bars represent 1000 μm (large hippocampal image) and 40 μm (inset). In panel (B), scale bars represent 1000 μm (large image) and 60 μm (inset).

3.3.4 Deficiency of ABCA1 reduces soluble apoE levels *in vivo*

We and others have previously shown that ABCA1 is required to maintain normal levels of apoE in the CNS [42, 43]. Western blots were used to determine if PBS-extractable apoE levels were decreased as expected in the presence of the APP or PS1 transgenes. In 10 month-old Tg-SwDI/B mice, PBS-soluble apoE levels were reduced by approximately 75% in APP+/ABCA1-/- hippocampus compared to APP+/ABCA1+/+ controls ($p<0.001$, Figure 3-6A). Similarly, in 12 month-old APP/PS1 mice, soluble apoE levels were 80% lower in hippocampus of mice lacking ABCA1 compared to animals with wild-type levels of ABCA1 (Figure 3-6B). Mice with heterozygous levels of ABCA1 had normal levels of soluble apoE (Figure 3-6B). Similar results were observed in the cortex of APP/PS1 mice (data not shown). These observations confirm our previous results and demonstrate that the influence of ABCA1 on apoE levels in brain is maintained in the presence of the APP and PS1 transgenes.

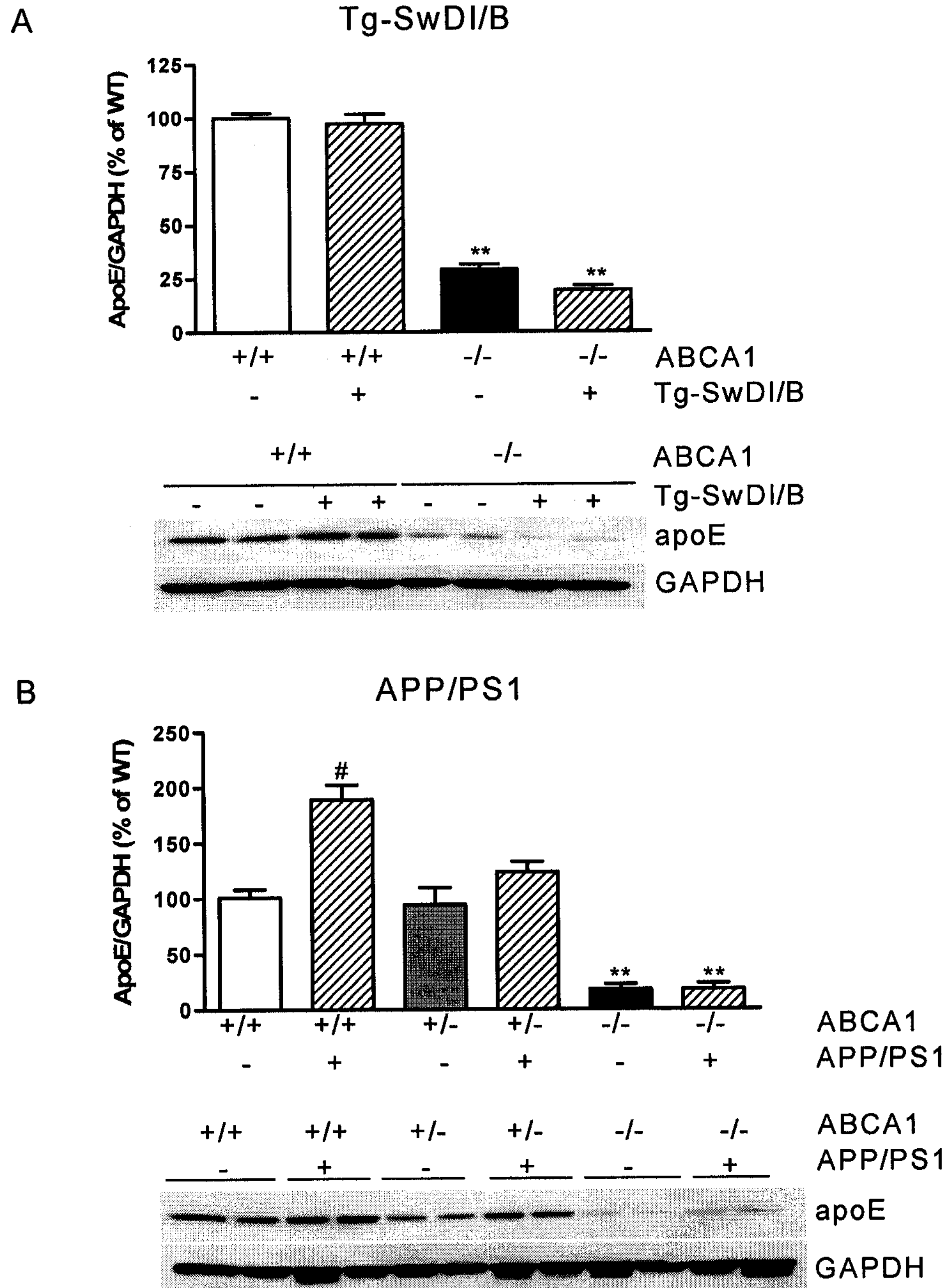

Figure 3-6: ABCA1 deficiency decreases soluble apoE levels in cortex and hippocampus.

PBS-extractable apoE levels were determined by Western blot in hippocampus of Tg-SwDI/B (A) and APP/PS1 (B) mice and quantified by densitometry. Graphs are expressed as % of wild-type (APP-ABCA1+/+ mice were assigned a 100%) and illustrate at least two independent experiments. (A) Data corresponds to eight individual mice for each genotype [4F, 4M]. (B) Data represents APP-, ABCA1+/+ (n=6 [3F, 3M]), APP+, ABCA1+/+ (n=5 [2F, 3M]), APP-, ABCA1+/- (n=6 [3F, 3M]), APP+, ABCA1+/- (n=6 [2F, 4M]), APP-, ABCA1-/- (n=4 [2F, 2M]) and APP+, ABCA1-/- (n=5 [2F, 3M]) mice. Western blots show two representative samples per group. GAPDH was used as an internal loading control. ** represents $p<0.001$ compared to wild-type (APP-, ABCA1+/+) control and # represents $p<0.05$ compared to non-transgenic animals by ANOVA with Newman-Keuls post test.

ApoE levels have been reported to be elevated in response to chronic or acute neuronal damage [30-33]. We observed elevated levels of soluble apoE in APP/PS1 transgenic mice compared to non-transgenic controls that contained at least one functional copy of *ABCA1* (Figure 3-6B), but did not observe this effect in Tg-SwDI/B transgenic mice (Figure 3-6A). This could be due to differences in the degree of AD pathology in each model at the time of assessment, including less amyloid deposition in Tg-SwDI/B compared to APP/PS1 mice and different distribution of amyloid, with Thioflavine-S positive deposits being mostly parenchymal in the APP/PS1 and vascular in the Tg-SwDI/B animals.

In the APP/PS1 model with extensive Aβ deposition, APP/PS1 transgenic mice with wild-type levels of ABCA1 showed a significant increase in PBS-extractable apoE in hippocampus (90%, $p<0.001$) compared to ABCA1+/+ mice lacking the APP/PS1 transgenes (Figure 3-6B). ABCA1 heterozygous mice carrying the APP/PS1 transgenes also showed a trend toward increased apoE levels in the hippocampus (30%, $p>0.05$) compared to ABCA1+/- mice lacking the APP and PS1 transgenes (Figure 3-6B). Similar results were observed in cortex of APP/PS1 mice (data not shown). These observations suggest that apoE expression is induced in mice with abundant Aβ deposition, consistent with a role for apoE in mitigating neuronal damage. Notably, apoE failed to be induced in the absence of ABCA1 in either model, suggesting that ABCA1 is required to observe the increase in apoE secretion under conditions of neuronal stress.

3.3.5 Deficiency of ABCA1 increases amyloid deposition in Tg-SwDI/B mice

In both Tg-SwDI/B and APP/PS1 models, guanidine-extractable Aβ levels were unchanged in the absence of ABCA1, suggesting that ABCA1 has minimal impact on the steady-state levels

of Aβ. Furthermore, both models exhibited a robust decrease in soluble apoE levels. Based on these observations, we expected to observe less amyloid deposition in ABCA1-deficient mice, consistent with previous observations that the level of apoE is a key determinant of amyloid deposition in murine models [36, 72]. However, contrary to our expectations of reduced amyloid burden, quantitative stereological assessment of amyloid in the hippocampus of 10 month-old Tg-SwDI/B mice revealed a significant 2-fold increase of amyloid in the absence of ABCA1, particularly in the subiculum and polymorph layer of the dentate gyrus ($p=0.02$) (Figure 3-7A, Table 3-1). Deposits in the subiculum were observed to be mostly vascular whereas the deposits in the dentate gyrus were predominantly parenchymal. These observations are consistent with an increase in deposited Aβ in Tg-SwDI/B mice that lack ABCA1. We also examined the impact of ABCA1 on amyloid deposits in the thalamus, which is a heavily vascularized region. Thalamic amyloid deposits were also significantly increased by 2-fold in the absence of ABCA1 ($p<0.005$, Figure 3-7B). Vascular deposits involved larger vessels in ABCA1 deficient mice compared to wild-type controls.

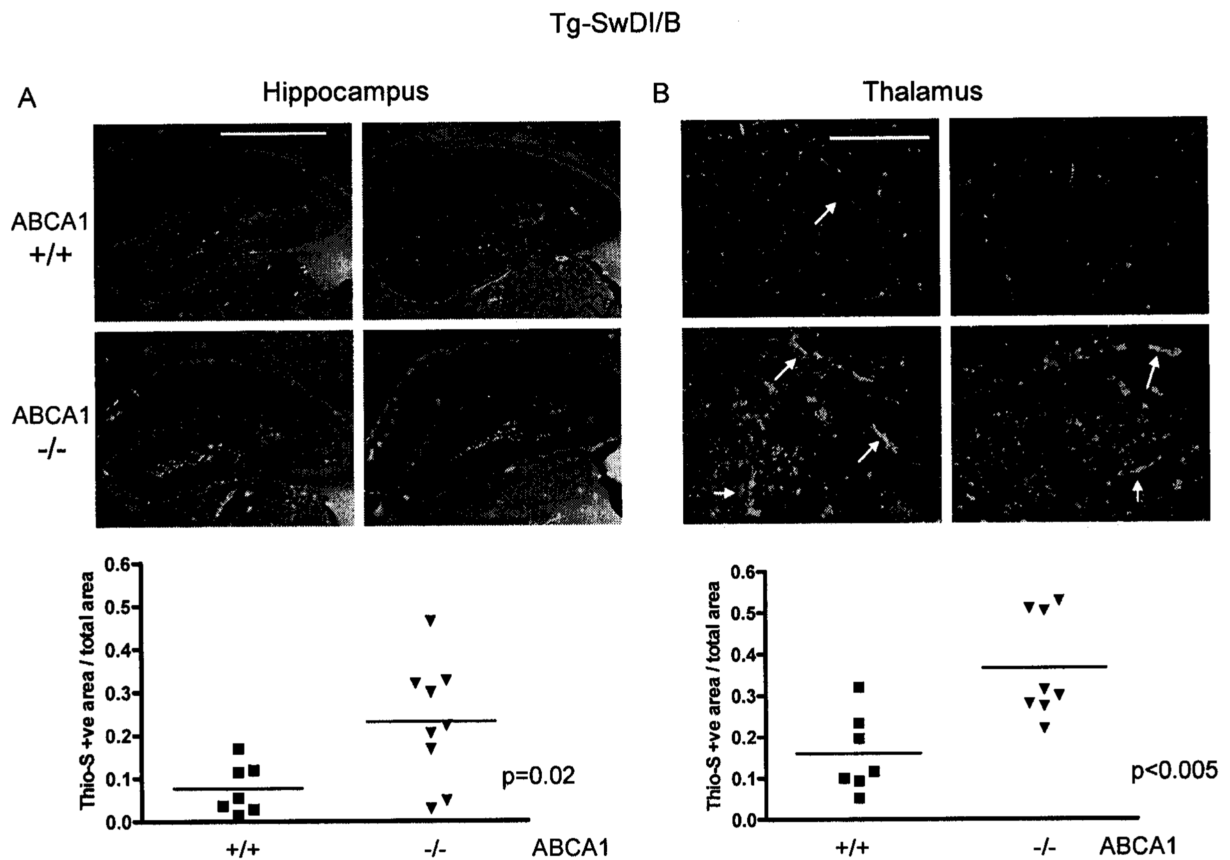

Figure 3-7: Absence of ABCA1 increases amyloid deposition in Tg-SwDI/B mice.

Thioflavine-S staining of hemicoronal sections from Tg-SwDI/B hippocampus (A) and thalamus (B) in the presence and absence of ABCA1. Pictures show two individual mice per genotype and correspond to 2.5x images for the hippocampus (A) and 10x images for thalamus (B). Scale bars represent 1000 µm in (A) and 250 µm in (B). Arrows point towards vessels. Amyloid load was quantified as described in methods in Tg-SwDI/B+, ABCA1+/+ (n=7 [3F, 4M]) and Tg-SwDI/B+, ABCA1-/- (n=8 [4F, 4M]) mice. Student's t-test was used for statistical analysis.

3.3.6 Amyloid burden is not diminished despite reduced apoE levels in APP/PS1 mice

Amyloid deposition was also assessed in the hippocampus and cortex of APP/PS1 mice between 12-13 months of age, a time in disease progression characterized by abundant amyloid deposits in this model. Again contrary to our expectations of reduced amyloid due to lower apoE levels, amyloid burden was not significantly diminished in either the hippocampus (Figure 3-8A, B, and Table 3-1) or cortex (Figure 3-8C, D, and Table 3-1) of wild-type,

heterozygous, and ABCA1-deficient mice. These findings are consistent with no significant change in Aβ deposits in APP/PS1 mice that lack ABCA1. However, unlike the Tg-SwDI/B model, we observed that deletion of ABCA1 resulted in no significant increase in amyloid burden or distribution in APP/PS1 mice when quantified either by fluorescence threshold per unit area (Figure 3-8, Table 3-1), or by plaque number per unit area (Table 3-1).

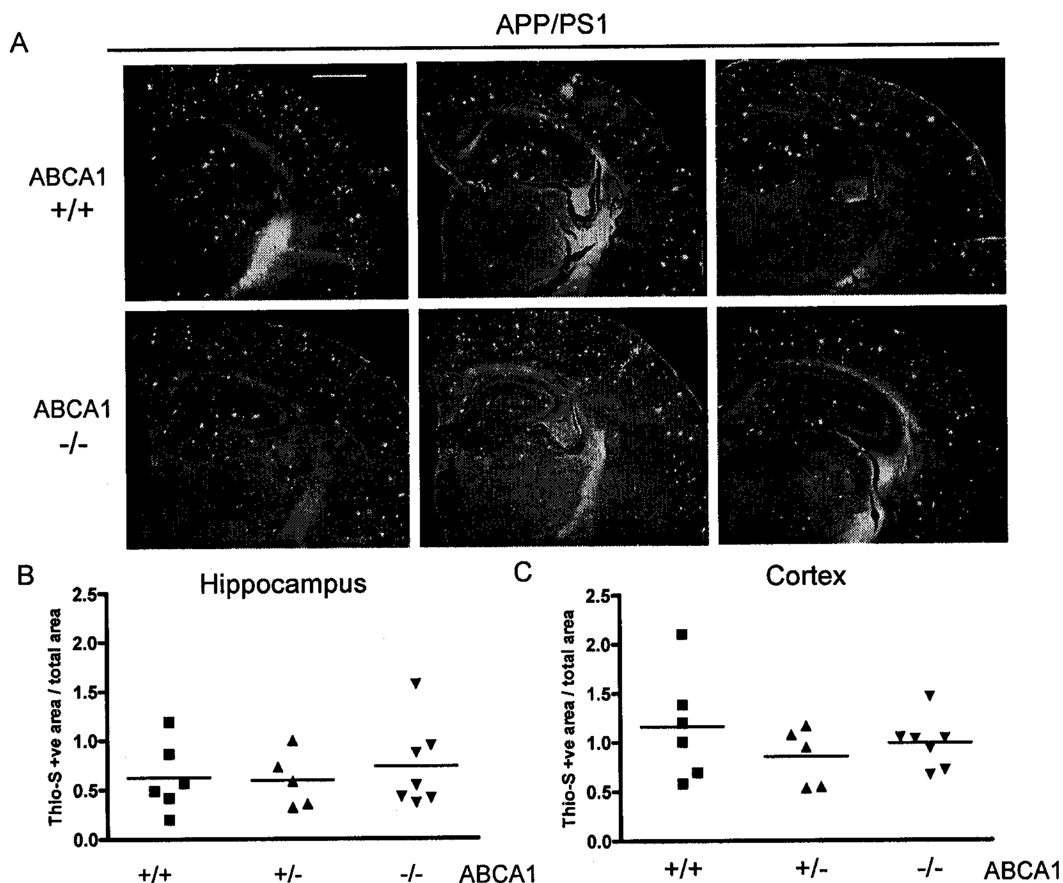

Figure 3-8: Absence of ABCA1 does not diminish amyloid burden in APP/PS1 mice.

(A) Thioflavine-S staining of hemicoronal sections from APP/PS1 mice in the presence and absence of ABCA1. Scale bars represent 1000 µm. (B) Amyloid load was quantified in the hippocampus (B) and cingulate cortex (C) as described in methods. The graph corresponds to APP/PS1+, ABCA1+/+ (n=6 [2F, 4M]), APP/PS1+, ABCA1+/- (n=5 [2F, 3M]) and APP/PS1+, ABCA1-/- (n=7 [3F, 4M]) mice. No significant differences were found by ANOVA.

3.3.7 ABCA1 regulates apoE solubility in brain

Previous studies have shown that insoluble, non-PBS extractable apoE appears in transgenic AD mice during the development of amyloid plaques [73], suggesting that apoE becomes sequestered within plaques and is removed from the soluble pool. To determine whether the absence of ABCA1 affected this insoluble pool of apoE, we analyzed the guanidine-extractable pool of apoE by Western blot.

In Tg-SwDI/B mice, guanidine-soluble apoE levels were reduced only by 50% in the hippocampus of ABCA1-deficient mice compared to mice with wild-type levels of ABCA1 ($p<0.01$, Figure 3-9). Because deficiency of ABCA1 resulted in a 75% reduction in soluble apoE levels, these observations suggest apoE may be beginning to be sequestered in amyloid plaques at this stage in pathogenesis.

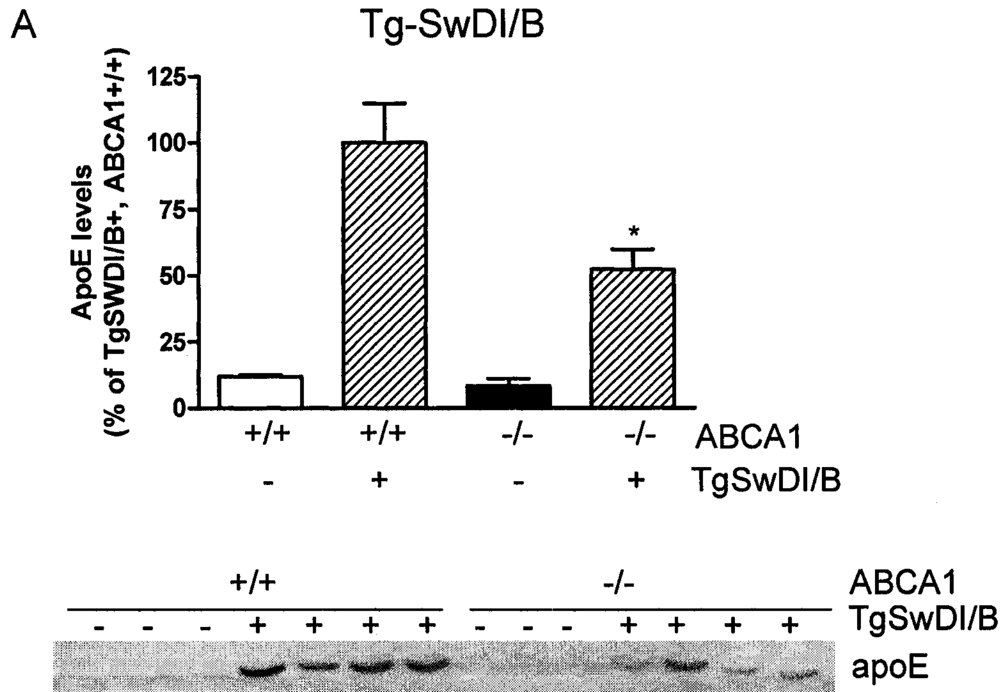

Figure 3-9: ABCA1-deficient Tg-SwDI/B mice have more insoluble apoE than expected.

Guanidine-soluble apoE levels were determined by Western blot in the hippocampus of Tg-SwDI/B-, ABCA1+/+ (n=6 [3F, 3M]), Tg-SwDI/B+, ABCA1+/+ (n=9 [5F, 4M]), Tg-SwDI/B-, ABCA1-/- (n=6 [3F, 3M]) and Tg-SwDI/B+, ABCA1+-/- (n=8 [4F, 4M]) mice and quantified by densitometry. Coomasie blue staining of parallel gels was used to normalize for protein loading. Graphs are expressed as % of APP+, ABCA1+/+ (these samples were assigned to 100%) and illustrate four independent experiments. Western blots show representative samples. * represents $p<0.01$ compared to transgenic ABCA1 wild-type (APP+, ABCA1+/+) control by ANOVA with Newman-Keuls post test.

In APP/PS1 mice with abundant plaques, we observed a clear ABCA1-dose dependent increase in insoluble apoE levels in both hippocampus and cortex (Figure 3-10). Compared to mice with wild-type levels of ABCA1, insoluble apoE was elevated by 63%, ($p>0.05$) in hippocampus and by 175% ($p>0.05$) in cortex in ABCA1+/- compared to ABCA1+/+ mice (Figure 3-10A and B respectively). This increase in insoluble apoE was even more dramatic in the complete absence of ABCA1 (Figure 3-10A, B), with a 2-fold increase observed in both hippocampus and cortex ($p<0.01$).

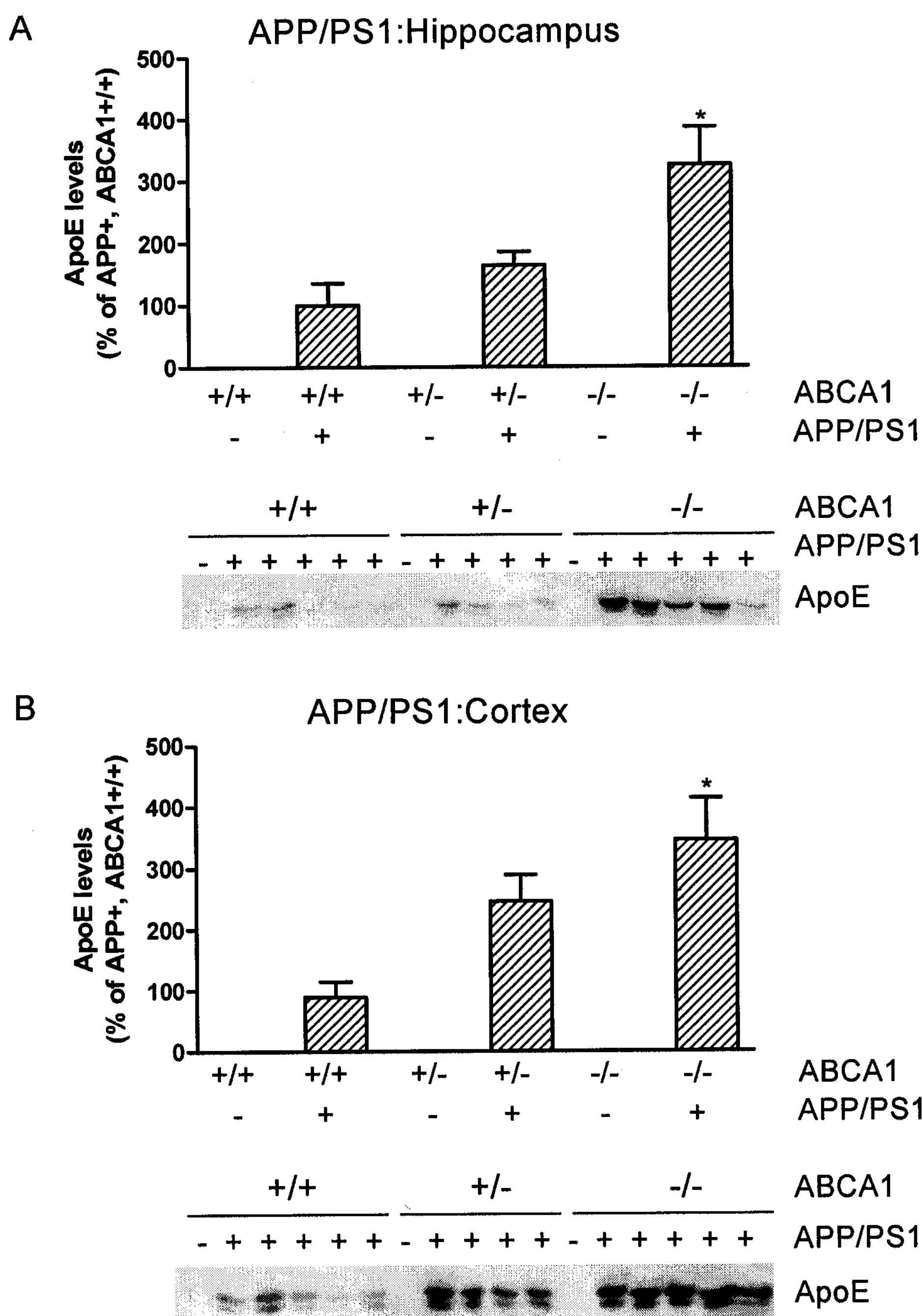

Figure 3-10: Insoluble apoE levels are increased in APP/PS1 ABCA1-deficient mice:

Guanidine-soluble apoE levels were determined by Western blot in the hippocampus (A) and cortex (B) of APP-, ABCA1+/+ (n=2 [1F, 1M]), APP+, ABCA1+/+ (n=5 [2F, 3M]), APP-, ABCA1+/- (n=2 [1F, 1M]), APP+, ABCA1+/- (n=4 2F, 2M]), APP-, ABCA1-/- (n=3 [1F, 2M]) and APP+, ABCA1-/- (n=5 [2F, 3M]) mice and quantified by densitometry. Coomasie blue staining of parallel gels was used to normalize for protein loading. Graphs are expressed as % of APP+, ABCA1+/+ (these samples were assigned to 100%) and illustrate at least two independent experiments. Western blots show representative samples. * represents p<0.01 compared to transgenic ABCA1 wild-type (APP+, ABCA1+/+) control by ANOVA with Newman-Keuls post test.

These observations demonstrate that ABCA1 has a marked effect on the distribution of apoE in the brain. In the Tg-SwDI/B model, lack of ABCA1 decreases the proportion of insoluble apoE in parallel with the decrease in soluble apoE. However, the decrease in the insoluble fraction of apoE is less than expected. In the APP/PS1 model, lack of ABCA1 results in a dramatic increase in the proportion of apoE found in the insoluble fraction. This observation suggests that absence of ABCA1 shifts the distribution of apoE from soluble to insoluble pools during the formation of amyloid plaques *in vivo*.

3.4 Discussion

Much current research supports a role for cholesterol in AD [19, 74], suggesting that genes that regulate cholesterol metabolism may influence the pathogenesis of AD. The cholesterol and phospholipid transporter ABCA1 is a critical regulator of HDL metabolism in peripheral tissues, and is expressed in the brain where it has recently been shown to regulate apoE levels [42, 43]. ABCA1 has also been implicated in Aβ metabolism, and genetic studies suggest that polymorphisms in *ABCA1* may be associated with AD [75-77]. These observations indicate that *ABCA1* may be a gene of interest for AD, although whether ABCA1 influences AD neuropathology *in vivo*, and whether this occurs through effects on Aβ or apoE metabolism, have not yet been addressed. We, as well as two other groups [78, 79] now report *in vivo* studies supporting a prominent role for ABCA1 in amyloidogenesis.

In this study, we investigated the impact of ABCA1 deficiency on steady state Aβ levels, amyloid burden, and apoE abundance and distribution in the Tg-SwDI/B and APP/PS1 murine models of AD. Three major conclusions can be drawn from our study. First, the absence of ABCA1 did not significantly affect the levels of soluble or insoluble Aβ when crossed to either Tg-SwDI/B or APP/PS1 mice, nor did it alter the levels of endogenous murine Aβ in the

parental ABCA1-deficient animals. These observations suggest that ABCA1 may not play a major role in Aβ production *in vivo*. Second, ABCA1 is required to maintain apoE levels *in vivo*. This was previously shown in the parental ABCA1-/- mice [42, 43], and is here confirmed upon breeding ABCA1-deficient mice to either the Tg-SwDI/B or APP/PS1 models. This observation suggests that the effect of glial ABCA1 on secretion of apoE from astrocytes and microglia remains similar across a variety of genetic backgrounds and in the presence of APP and PS1 transgenes. Third, and in contrast to our expectations, the 'ABCA1 deficiency'-mediated decrease of apoE levels failed to reduce amyloid and Aβ deposition in both the Tg-SwDI/B and APP/PS1 models. Based on the lack of effect of ABCA1 on Aβ levels but the pronounced effect of ABCA1 on apoE levels *in vivo*, we predicted amyloid deposition would be decreased in the absence of ABCA1, in line with previous observations that apoE levels determine the extent of amyloid deposition *in vivo* [36-39]. In contrast, we observed more extensive parenchymal and vascular Aβ deposition and significantly elevated amyloid burden in the Tg-SwDI/B model despite a 75% reduction in apoE levels. In the APP/PS1 model, we again observed no decrease in amyloid burden despite an 80% decrease in soluble apoE levels in the absence of ABCA1.

One possible explanation for this relates to the role of apoE in Aβ clearance. Several studies have shown that apoE binds Aβ and suggested that this interaction might aid in the clearance of Aβ [80-83]. Furthermore, two recent studies have shown that astrocytes are capable of clearing Aβ deposits from brain slices of aged APP transgenic mice [84, 85] and that this process is apoE-dependent [86]. Together, these observations suggest that apoE is a key factor in regulating Aβ clearance *in vivo*. It is plausible to hypothesize that the low levels of apoE that exist in the brains of ABCA1-deficient mice are sufficient to allow for amyloid formation, but not enough to mediate effective Aβ clearance.

It is also possible that the poor lipidation of apoE in the absence of ABCA1 renders apoE prone to sequestration in amyloid plaques and enhances the conversion of Aβ from soluble peptides into amyloid. This hypothesis is supported by our observation that ABCA1 markedly affects the distribution of apoE in soluble compared to insoluble pools. In the Tg-SwDI/B model, ABCA1 deficiency reduced soluble apoE levels by 75% but reduced insoluble apoE levels only by 50% compared to mice with ABCA1. In the APP/PS1 model, soluble apoE was decreased by 80%, but apoE extracted from the insoluble fraction was elevated 2-fold in ABCA1-/- compared to ABCA1+/+ mice. Wahrle *et al* also observed increased levels of guanidine-extractable insoluble apoE in transgenic PDAPP mice [79].

One limitation of our study is that the Western blot methodology used in our experiments does not allow us to accurately quantitate the relative proportion of insoluble compared to soluble apoE. Furthermore, the two AD models used in this study cannot be directly compared, due primarily to differences in the stage of disease pathogenesis and Aβ properties. Although the Tg-SwDI/B and APP/PS1 mice were examined at similar ages, the APP/PS1 model exhibited a 10-fold greater amyloid burden at the time of analysis compared to the Tg-SwDI/B animals. Additionally, Tg-SwDI/B mice express a human Aβ carrying the Dutch and Iowa mutations that may affect APP processing and influence the mechanism of Aβ deposition, whereas the APP/PS1 model generates wild-type human Aβ. These important differences in Aβ species in these two models could potentially alter binding to and deposition/clearance with apoE. Nevertheless, our observation that nearly all detectable apoE was present in the insoluble fraction in the APP/PS1 model raises the possibility that apoE continues to be recruited to amyloid plaques after they are seeded, and that the poorly lipidated apoE present in the brains of ABCA1-/- mice may either be recruited more effectively to amyloid deposits or is unable to clear these deposits as efficiently as normally lipidated apoE.

One of the strengths of this study is that we used two independent models of AD with different transgenes, promoters, and genetic backgrounds. The conclusions that deficiency of ABCA1 has no effect on soluble or guanidine-extractable steady state Aβ levels, but significantly decreases apoE levels and fails to diminish amyloid burden are consistent in both these models, despite the important differences in Aβ species generated in the Tg-SwDI/B compared to APP/PS1 mice. Our major conclusions that deficiency of ABCA1 is associated with low levels of apoE, a shift in the distribution of apoE from soluble to insoluble pools, and either no change or increased deposition of fibrillar Aβ are also similar to those of Koldamova *et al* and Wahrle *et al* [78, 79]. Using APP23 mice, Koldamova *et al* report that the absence of ABCA1 results in significantly decreased apoE levels, significantly increased formic-acid extractable Aβ levels, and significantly increased parenchymal and vascular amyloid deposition [78]. Using the PDAPP model, Wahrle *et al* observed that deficiency of ABCA1 leads to significantly elevated guanidine-extractable Aβ levels, a trend toward increased parenchymal and vascular deposition of Aβ and apoE, and a dramatic shift of apoE distribution from the soluble to the guanidine-extractable fraction [79]. Taken together, three laboratories have now independently demonstrated that amyloid deposition fails to be reduced in four models of AD despite low apoE levels in the absence of ABCA1, and that these effects extend across differences in the transgene expressed, the mutations it carries, the promoter used, and genetic background of the animals.

One difference among the three studies is in the effect of ABCA1 on Aβ levels. Koldamova *et al* found that lack of ABCA1 led to a trend toward increased soluble Aβ and a significant elevation of insoluble Aβ extracted using formic acid [78]. Similarly, Wahrle *et al* report that the levels of soluble and guanidine-extractable Aβ are elevated in the absence of ABCA1 [79]. Using a different human-specific ELISA assay, we observed no differences in either soluble or guanidine-extractable Aβ levels between ABCA1-deficient and wild-type brains from either the

Tg-SwDI/B or APP/PS1 models. It is possible that the guanidine extraction protocol used in our study may have failed to quantitatively extract some of the highly aggregated Aβ sequestered in plaques compared to formic acid extracts [86]. Additionally, compared to the wild-type human Aβ generated in the APP/PS1 model, the mutations present in the Aβ of Tg-SwDI/B mice might interfere with its detection by ELISA, lowering the sensitivity of the test and obscuring possible increases in the insoluble fraction of Aβ. Differences in Aβ detection properties might also help to explain why we observed more deposited Aβ using immunohistochemistry in ABCA1-deficient Tg-SwDI/B mice compared to controls, yet did not detect elevated Aβ levels by ELISA.

A second difference among the studies relates to the proportion of apoE residing in the soluble or insoluble fractions. Koldamova *et al* did not detect elevated levels of insoluble apoE after formic acid extraction [78], whereas we and Wahrle *et al* both observed accumulation of apoE in the guanidine-extractable fraction [79]. Furthermore, Wahrle *et al* demonstrated that this apoE co-localized with amyloid [79].

The mechanisms underlying the shift in apoE distribution in soluble to insoluble fractions remain to be elucidated. The low density lipoprotein receptors LDLR and LDLR-related protein are the major apoE receptors in the CNS [87-89], and LRP is postulated to mediate clearance of Aβ:apolipoprotein complexes [89]. Because delipidated apoE binds LRP poorly [88, 90], and because the lipidation status of apoE also influences its interactions with Aβ [81, 91, 92], it is possible that that the poorly lipidated apoE that exists in the brains of ABCA1-deficient mice [42, 43] may interact differently with Aβ and the resulting complexes may not be efficiently recognized by LRP. Together, these mechanisms could lead to delayed clearance and increased deposition of Aβ.

Given the large number of studies demonstrating that intracellular cholesterol levels influence APP processing, an alternative explanation of our results is that an increase in Aβ production mediated by cholesterol-enriched ABCA1-deficient neurons may be precisely balanced by diminished Aβ and amyloid deposition resulting from ABCA1-mediated reductions in apoE levels. Because we observed no change in Aβ □levels in our study, we did not measure Aβ production directly by testing for changes in APP CTF levels. Notably, despite detecting an increase in Aβ □levels, Aβ production was observed to be unaffected in the absence of ABCA1 in the APP23 and PDAPP models [78, 79]. However, because our study, as well as those of Koldamova *et al* and Wahrle *et al*, used total ABCA1-deficient mice, elucidating the *in vivo* impact of ABCA1 on Aβ production independently of its effect on glial-derived lipoprotein homeostasis will require additional investigations in neuronal- and glial-specific ABCA1 knockout mice.

Whether the prevalence of AD is increased in TD patients that lack ABCA1 has not been specifically addressed, primarily because TD is a rare disease and most patients do not survive past 70 years of age [93]. To our knowledge, there is only one case report of a proband with a compound mutation in the *ABCA1* gene who developed and died of complications related to cerebral amyloid angiopathy (CAA) [94]. Three studies have investigated whether single nucleotide polymorphisms in the *ABCA1* gene are associated with AD [75-77]. Wollmer *et al* reported that the R219K gain-of-function single nucleotide polymorphism in *ABCA1* (R219K) has been suggested to delay the age of onset of AD by 1.6 years [75]. Katzov *et al* reported significant associations with the R219K, R1587K, and V771M on AD in single marker and haplotype analyses conducted on European subjects [76]. However, these findings were not replicated in a large case-control study of North-American subjects using either single marker or haplotype analyses [77]. Further investigations will therefore be required to determine whether inactivation of ABCA1 affects AD in humans. Nevertheless, the results of our study, together

with Koldamova and Wahrle [78, 79], suggest that the impact of ABCA1 on AD may largely be mediated through its effect on apoE levels and/or lipidation status.

3.5 Reference list

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Chapter 4 - Expression of human *ABCA1* in bacterial artificial chromosome transgenic mice does not mitigate Alzheimer neuropathology *in vivo.**

4.1 Introduction

Apolipoprotein E (apoE) is a well-validated risk factor for late-onset Alzheimer Disease (AD)[1]. In the central nervous system (CNS), apoE is secreted by astrocytes and microglia and serves as the major cholesterol carrier in brain [2]. ApoE also binds Aβ and is found in amyloid plaques [3, 4]. ApoE-deficient mice deposit Aβ but not amyloid, [5, 6], and the extent of amyloid deposition correlates with apoE gene dose [7]. These findings suggest that factors that regulate apoE abundance may affect amyloidogenesis.

We and others have shown that the ATP-binding cassette transporter ABCA1 modulates CNS apoE levels [8-10]. ABCA1 effluxes cellular lipids onto lipid-poor apolipoprotein acceptors [11], and deficiency of ABCA1 results in nearly undetectable plasma high-density lipoprotein (HDL) levels, impaired cholesterol efflux, and an increased risk of cardiovascular disease [12-14]. In the brain, ABCA1 is expressed in neurons, astrocytes, and microglia, and is induced by Liver X Receptor (LXR) and Retinoic X Receptor (RXR) agonists [15-18]. ABCA1-deficient glia secrete less apoE than wild-type cells and are impaired in cholesterol efflux to apoE [8]. These effects may underlie the drastic reduction in CNS apoE levels found in ABCA1-deficient mice and explain the poor lipidation of the remaining apoE particles [8-10].

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Recently, we and others demonstrated that ABCA1 modulates amyloid deposition *in vivo* [19-21]. Because apoE levels directly correlate with the extent of amyloid burden, the reduced levels of apoE in ABCA1-deficient mice led to the prediction that fewer amyloid deposits would be observed in the absence of ABCA1. In contrast, ABCA1-deficient mice developed an equal or greater amyloid burden than wild-type mice in a total of four independent models of AD [19-21], demonstrating that low levels of poorly-lipidated apoE promote as much amyloid deposition as wild-type levels of normally-lipidated apoE. These observations raise the converse possibility that ABCA1 overexpression may reduce amyloid levels.

We therefore crossed APP/PS1 mice to *ABCA1* bacterial artificial chromosome (BAC) transgenic mice, which express human *ABCA1* from endogenous regulatory signals [22]. BAC transgenic models are particularly desirable for *in vivo* investigations, as the transgene is expressed from physiologically relevant regulatory elements that conserve appropriate developmental and tissue-specific expression patterns [23]. Accordingly, the expression pattern of human *ABCA1* from a BAC transgene has been shown to mirror that of endogenous murine *abca1* in multiple tissues including brain [24] and protects from atherosclerosis in both whole-animal and bone marrow transplant paradigms [25, 26].

Here we report that although the *ABCA1* BAC leads to elevated ABCA1 and apoE levels in brain, this is only observed in animals without amyloid deposits. Despite clearly detectable human and murine ABCA1 mRNA in the brains of *ABCA1* BAC transgenic animals with AD neuropathology, ABCA1 protein levels fail to be correspondingly increased and no change in amyloid, apoE, or Aβ levels were found. These observations suggest that physiologically regulated ABCA1 in the AD brain may be subject to mechanisms that blunt its ability to attenuate amyloid deposition *in vivo*.

4.2 Experimental procedures

4.2.1 Animals

ABCA1 BAC transgenic mice containing BAC RP11-32H03 have been described previously [27]. APP/PS1 (line 85) mice (Jackson Laboratories, Bar Harbor) express a chimeric mouse/human APP650 cDNA containing the Swedish (KM670/671NL) mutation cointegrated with the human presenilin 1 (PS1) gene containing the DeltaE9 mutation [28]. APP/PS1 mice were crossed to *ABCA1* BAC transgenic animals, and double ABCA1/APP/PS1 transgenic animals were compared to APP/PS1 littermate controls. Animals were maintained on a chow diet (PMI LabDiet 5010), and all animal procedures were in accordance with the Canadian Council of Animal Care and the University of British Columbia Committee on Animal Care.

4.2.2 Neuropathological analyses

Thioflavin-S staining of amyloid plaques was performed and quantified as described [19]. Human Aβ levels were quantified by ELISA (Biosource, Camarillo) and normalized to total protein.

4.2.3 Protein extraction and Western blot

Tissue extractions were performed as described previously [19]. Briefly, brain regions were homogenized in ice-cold phosphate buffered saline (PBS) containing Complete protease inhibitor (Roche, Mississauga) in a Tissuemite homogenizer followed by centrifugation. The supernatant (soluble fraction) was then removed and used to evaluate PBS-extractable apoE. The pellets from the PBS solubilization step were resuspended in ice-cold lysis buffer containing 10% glycerol, 1% TritonX-100 and Complete protease inhibitor (Roche, Mississauga) in PBS and centrifuged in order to extract ABCA1. The pellet from this step (insoluble fraction) was finally solubilized in 5 M guanidine hydrochloride in 50 mM Tris–HCl, pH 8.0 in order to evaluate plaque-associated Aβ. Brain tissues from all animals were extracted in an identical manner, and all fractions were immediately frozen at -80°C until analysis.

Protein concentrations were determined by DC Protein Assay (BioRad, Hercules). For Western blots, tissue lysates were resolved by SDS-PAGE and immunodetected using a monoclonal anti-ABCA1 antibody (AC10) [16], or an anti-GAPDH antibody (Chemicon, Temecula) as a loading control. Blots were developed using enhanced chemiluminescence (Amersham, Piscataway) and quantified using NIH Image J.

4.2.4 ApoE measurements

Murine apoE levels were determined by ELISA as previously described [9]. Plates were coated with anti-apoE (WU E-4). Samples were diluted in 0.5% BSA, 0.025% Tween-20 in PBS. Standards were based on plasma from Swiss-Webster rats containing 61.7 μg/ml apoE [9]. Following an overnight incubation at 4°C, plates were incubated with goat anti-apoE (EMD Biosciences, San Diego) followed by biotinylated anti-goat antibody (Vector Laboratories, Burlington). Plates were developed with poly-horseradish peroxidase streptavidin (Pierce, Rockford) and ultra-slow 3,3′,5,5′-tetramethylbenzidine (Sigma, St. Louis), stopped with 1N HCl and read at 450 nm.

4.2.5 Quantitative Reverse-Transcription PCR (qRT-PCR)

RNA was extracted using Trizol (Invitrogen, Burlington) and treated with DNAseI. cDNA was generated using oligo-dT primers and Taqman Reverse transcription reagents (Applied Biosystems, Foster City). Quantitative real-time PCR primers were designed using PrimerExpress (Applied Biosystems, Foster City) to span human- and murine-specific regions of ABCA1 or regions identical in both species. Primer sequences and cycling conditions are provided upon request. Real-time quantitative PCR was done with Sybr green reagents (Applied Biosystems, Foster City) on an ABI 7000 (Applied Biosystems, Foster City). Each

sample was assayed at least in duplicate, normalized to β-actin and analyzed with 7000 system SDS software v1.2 (Applied Biosystems, Foster City) using the relative standard curve method.

4.2.6 CSF cholesterol measurements

CSF was isolated as previously described [29]. Total cholesterol measurements were performed using the fluorogenic Amplex Red Cholesterol Assay Kit (Molecular Probes, Burlington).

4.2.7 Statistical analysis

Data is shown as mean ± standard error of the mean and analyzed by two-tailed unpaired Student's t-tests. Welch's correction for unequal variances was applied when variances were significantly different between groups. Analyses were performed using Graphpad Prism (version 4.0, San Diego).

4.3 Results

4.3.1 ABCA1 and apoE levels are elevated in cortex of *ABCA1* BAC transgenic mice

To test whether excess ABCA1 can mitigate amyloid deposition, *ABCA1* BAC Tg mice were crossed to the APP/PS1 model of AD, resulting in four groups consisting of wild-type, *ABCA1* BAC, APP/PS1, and ABCA1/APP/PS1 animals. Importantly, these littermates are optimally matched for their mixed (75% C57Bl/6, 25% C3H) genetic background. To first validate the *ABCA1* BAC transgenic model, we confirmed that the presence of the *ABCA1* BAC transgene resulted in elevated ABCA1 levels in brain. Compared to wild-type animals, ABCA1 protein levels were increased by approximately 50% in *ABCA1* BAC Tg cortex and approximately 100 % in liver (cortex: $p=0.0009$, $N> 13$; liver: $p=0.0042$, $N=8$) (Figure 4-1A). These values represent the sum

of both human and murine ABCA1 protein, as our ABCA1 antibody does not distinguish between human and murine ABCA1 protein. That human *ABCA1* expression was considerably more robust in the liver compared to the brain suggests that the endogenous regulatory sequences present on BAC RP11-23H03 are less efficiently recognized in brain.

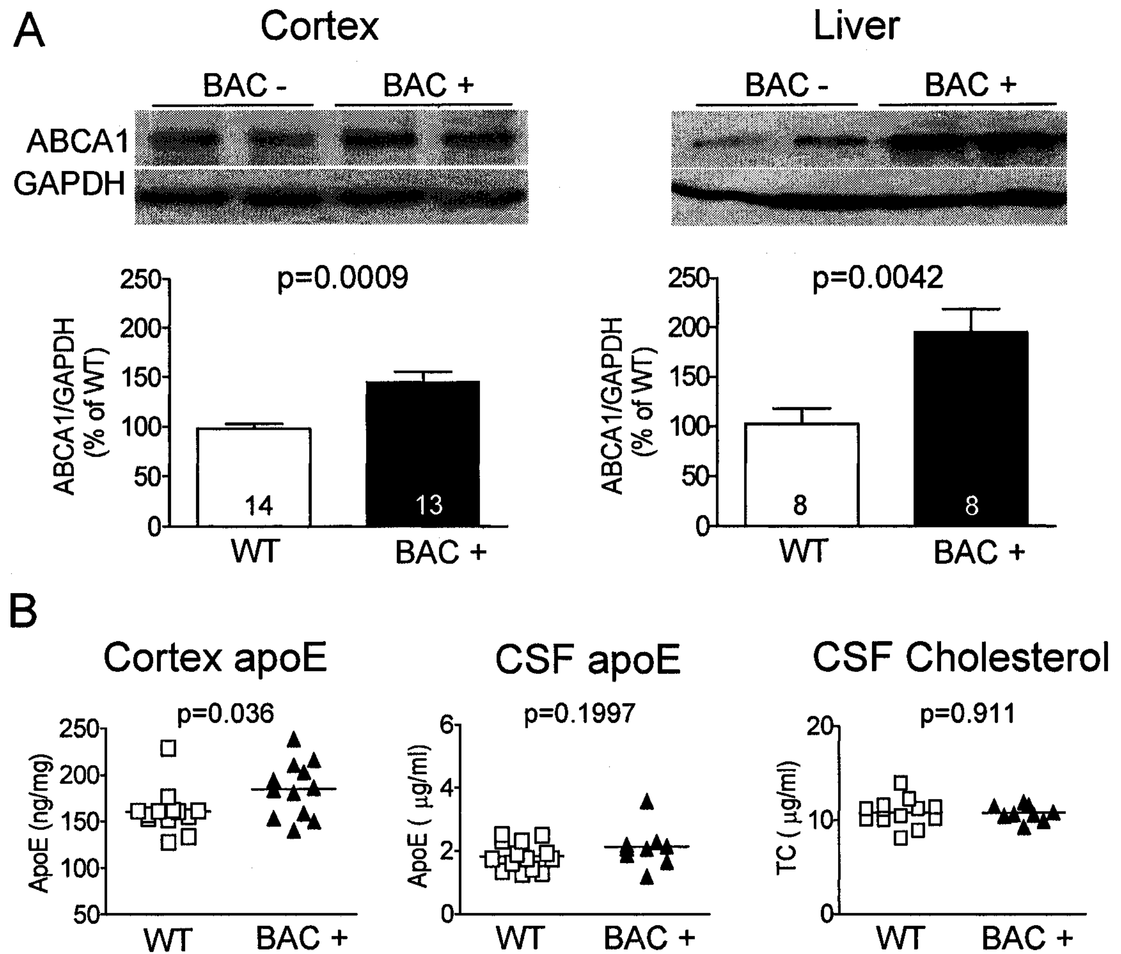

Figure 4-1: The *ABCA1* BAC increases cortical and liver ABCA1 and cortical apoE levels but does not affect CSF apoE or cholesterol

(A) Total ABCA1 protein in cortex and liver was measured by Western blot and quantitated by densitometry relative to GAPDH levels. Graphs are expressed as % of wild-type (wild-type animals were assigned a 100%) and illustrates the pooled results of four independent measurements of N=14 wild-type (WT) and N=13 *ABCA1* BAC transgenic (BAC+) cortical samples, and two independent measurements of N=8 wild-type and N=8 *ABCA1* BAC transgenic liver samples. (B) PBS-soluble cortical apoE levels were measured by ELISA and normalized for total protein. The graph represents two independent experiments of N=13 wild-type (WT) and N=12 *ABCA1* BAC transgenic (BAC+) samples measured in triplicate. (C) CSF apoE levels were assessed by ELISA. The graph represents N=14 wild-type (WT) and N=9 *ABCA1* BAC transgenic (BAC+) samples measured in duplicate. (D) CSF total cholesterol was analyzed using the Amplex Red Cholesterol Assay kit. The graph represents N=13 wild-type (WT) and N=9 *ABCA1* BAC transgenic (BAC+) samples measured in duplicate. Two-tailed student's t-tests were used for statistical analysis.

We next determined whether elevated brain ABCA1 resulted in increased cortical and cerebrospinal fluid (CSF) apoE levels and CSF cholesterol (Figure 4-1B). PBS-soluble apoE levels were significantly increased by 15% in the cortex of *ABCA1* BAC transgenic mice (mean apoE level = 185.1 ng/mg) when compared to wild-type controls (mean apoE level = 160.9 ng/mg) ($p=0.036$, $N>12$). However, no significant changes were observed in either CSF apoE levels or CSF cholesterol in the presence of ABCA1 overexpression. These data suggest that physiological overexpression of ABCA1 protein in the BAC transgenic model is sufficient to promote a subtle increase in apoE levels in brain tissue, but also indicates that the level of ABCA1 overexpression achieved in the BAC model may not be sufficiently robust to alter the levels or lipidation of circulating apoE in CSF, where particles are subject to remodeling.

4.3.2 Amyloid burden, Aβ and apoE levels are not changed by the presence of physiologically regulated human *ABCA1*

To determine whether excess ABCA1 may inhibit amyloidogenesis *in vivo*, we then analyzed APP/PS1 mice with and without excess ABCA1 at 13-16 months of age. No significant differences in amyloid burden, guanidine-extractable Aβ levels, soluble apoE levels, or CSF cholesterol were observed between ABCA1/APP/PS1 transgenic animals relative to APP/PS1 littermate controls (Figure 4-2). These data suggest that physiologically regulated expression of human *ABCA1* from endogenous regulatory elements present on BAC RP11-32H03 does not significantly affect human Aβ or mouse apoE metabolism *in vivo*.

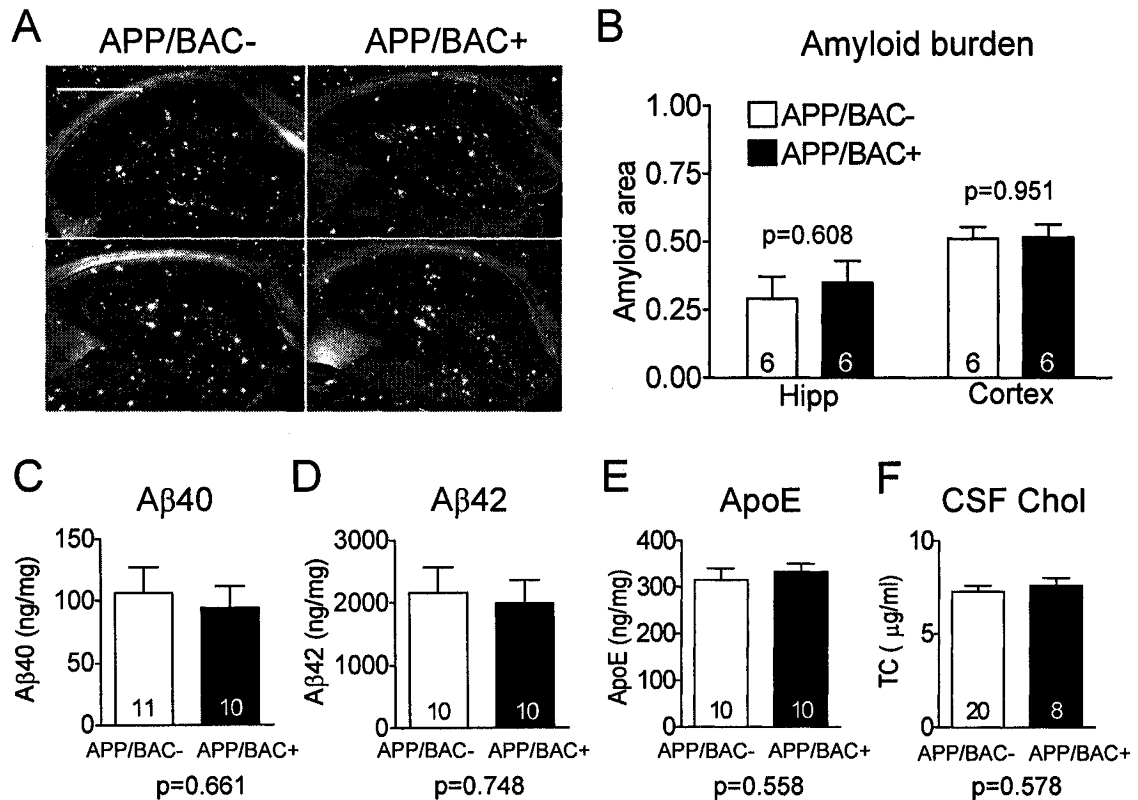

Figure 4-2: *ABCA1* BAC transgene expression has no significant effect on amyloid burden, Aβ level, apoE abundance, or CSF cholesterol

(A) Thioflavine-S staining of hemicoronal sections from APP/PS1 hippocampi in the presence (BAC+) and absence (BAC-) of the *ABCA1* BAC transgene. Pictures show two individual mice per genotype and correspond to 2.5x images of the hippocampus. The scale bar represent 1000 □m. Amyloid load was quantitated for hippocampus and cingulate cortex in N=6 APP/PS1 (BAC-) and N=6 ABCA1/APP/PS1 (BAC+) animals. (B) The graph is expressed as the percentage of Thioflavin-S positive area over total area. Guanidine-extractable Aβ40 (C) and Aβ42 (D) were measured by ELISA and normalized for total protein. The graphs correspond to N=11 APP/PS1 (BAC-) and N=10 ABCA1/APP/PS1 (BAC+) animals. (E) PBS-soluble cortical apoE levels were measured by ELISA and normalized for total protein. The graph represents two independent measurements (each performed in triplicate) of N=10 APP/PS1 (BAC-) and N=10 ABCA1/APP/PS1 (BAC+) animals. (F) CSF cholesterol levels were measured by Amplex Red assay in N=20 APP/PS1 (BAC-) and N=8 ABCA1/APP/PS1 (BAC+) mice. In all graphs, sample size is indicated by the number within the bar. Two-tailed student's t-tests were used for all statistical analysis.

4.3.3 Human *ABCA1* is expressed in brain and liver of ABCA1/APP/PS1 mice, but makes a significant contribution to total ABCA1 mRNA and protein levels only in liver

To determine whether the inability to detect a change in amyloid, Aβ , apoE or CSF cholesterol in the ABCA1/APP/PS1 mice could be attributed to an unexpected loss of human *ABCA1*

expression, qRT-PCR was used to quantify human, murine and total ABCA1 mRNA in cortex of ABCA1/APP/PS1 mice compared to APP/PS1 littermates. Validation tests first confirmed that the human primers were unable to amplify murine *abca1*, and that the murine primers did not amplify human *ABCA1*. Furthermore, the primers used to detect total ABCA1 mRNA amplified human and murine ABCA1 with equivalent efficiencies of 95.13% and 94.55%, respectively. As expected, human *ABCA1* mRNA is present only in animals carrying the *ABCA1* BAC transgene (Figure 4-3A). Furthermore, murine *abca1* mRNA levels are similar in ABCA1/APP/PS1 compared to APP/PS1 mice, showing that the presence of the human *ABCA1* BAC transgene has no significant influence on murine *abca1* mRNA levels (Fig 3B). Surprisingly, total ABCA1 mRNA levels in brain were indistinguishable between APP/PS1 and ABCA1/APP/PS1 mice, suggesting that although human *ABCA1* mRNA is detectable in brain, it has a negligible effect on total ABCA1 mRNA abundance (Figure 4-3C). Accordingly, we observed no increase in total ABCA1 protein levels in cortical extracts prepared from APP/PS1 and ABCA1/APP/PS1 mice (Figure 4-3D, E).

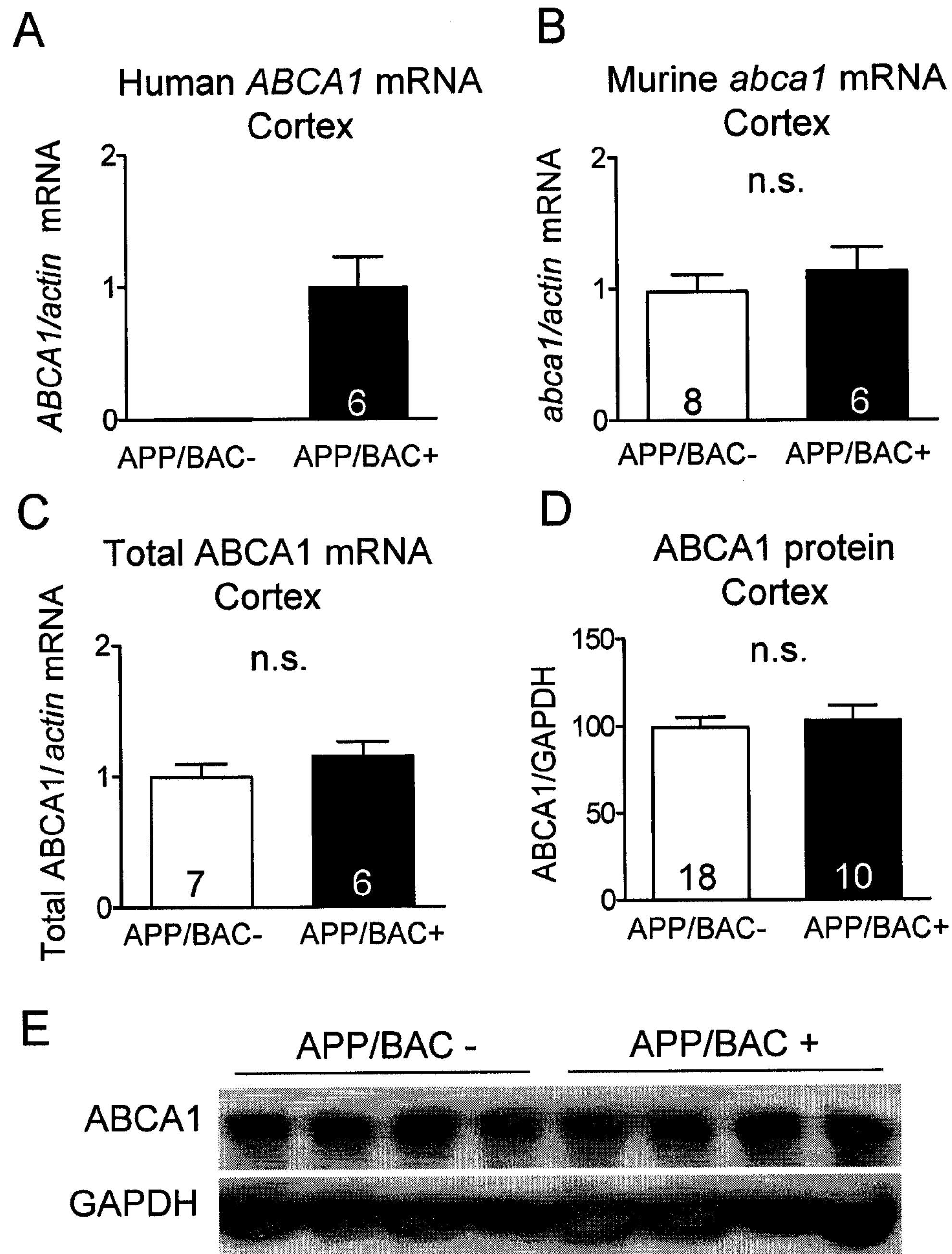

Figure 4-3: Human *ABCA1* expression in the cortex of APP/PS1 mice is insufficient to elevate total ABCA1 mRNA or protein levels.

ABCA1 mRNA levels were determined using QRT-PCR using (A) human-specific primers, (B) murine-specific primers, or (C) primers that do not distinguish between species. Graphs are expressed as fold difference of ABCA1/APP/PS1 (APP/BAC+) mice relative to APP/PS1 (APP/BAC-) controls, which were assigned an arbitrary value of one. Numbers in each bar represent the number of independent animals that were analyzed, using at least duplicate measurements per animal. (D, E) ABCA1 protein levels were evaluated by Western blot and normalized to GAPDH as an internal loading control from N≥10 independent mice per genotype. Two-tailed student's t-tests were used for all statistical analysis.

ABCA1 expression was also evaluated in the liver of these same mice. As expected, human *ABCA1* mRNA is detected only in animals carrying the *ABCA1* BAC transgene (Figure 4-4A), and murine *abca1* mRNA levels were no different between groups (Figure 4-4B). Unlike brain, however, total ABCA1 mRNA and protein levels were elevated approximately two-fold in ABCA1/APP/PS1 livers compared to APP/PS1 controls ($p=0.0345$, $N>5$ for mRNA, $p=0.0003$, $N=8$ for protein) (Figure 4-4D,E). These results demonstrate that the human BAC remains functional after breeding to APP/PS1 mice, but that its expression may be attenuated specifically in brain.

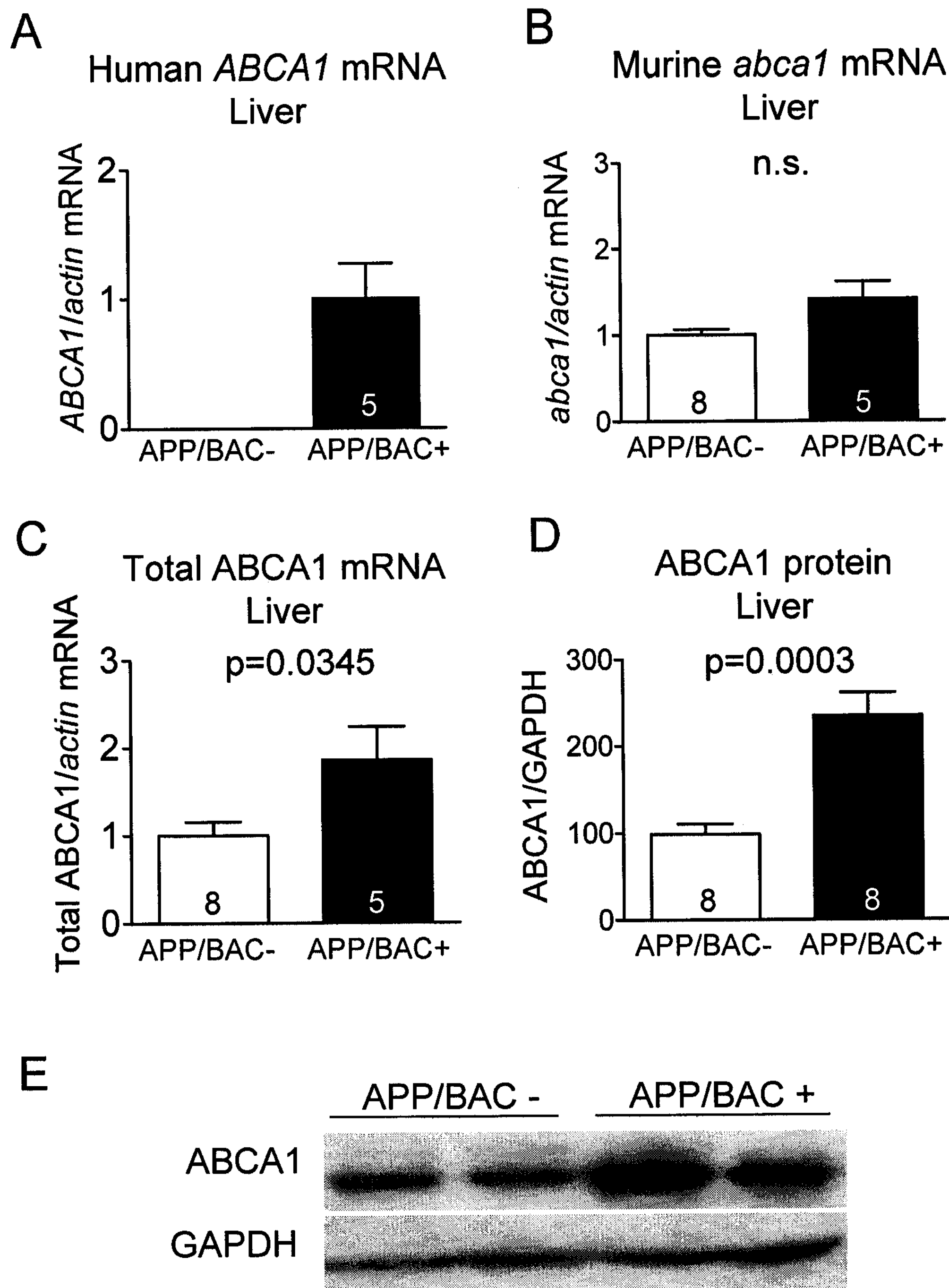

Figure 4-4: Human *ABCA1* expression in the liver of APP/PS1 mice results in a significant increase in total ABCA1 mRNA and protein levels.

ABCA1 mRNA levels were determined using QRT-PCR using (A) human-specific primers, (B) murine-specific primers, or (C) primers that do not distinguish between species. Graphs are expressed as fold difference of ABCA1/APP/PS1 (APP/BAC+) mice relative to APP/PS1 (APP/BAC-) controls, which were assigned an arbitrary value of one. Numbers in each bar represent the number of independent animals that were analyzed, using at least duplicate measurements per animal. (D, E) ABCA1 protein levels were evaluated by Western blot and normalized to GAPDH as an internal loading control from N= 8 independent mice per genotype. Two-tailed student's t-tests were used for all statistical analysis.

4.3.4 *ABCA1* mRNA but not protein levels are elevated in the amyloid-containing brain

To further explore these findings, we next compared ABCA1 mRNA and protein expression in cortex and cerebellum in littermate mice of all four genotypes. Compared to wild-type mice, murine *abca1* mRNA levels were significantly elevated in the cortex of APP/PS1 ($p<0.05$, $N=8$) and ABCA1/APP/PS1 ($p<0.05$, $n=6$) mice, but were unchanged in cerebellum ($p>0.05$, $N>7$) (Figure 4-5A). Similarly, human *ABCA1* mRNA levels were approximately three-fold higher in cortex ($p<0.05$, $N>6$) but not in cerebellum ($p>0.05$, $N>7$) of ABCA1/APP/PS1 compared to *ABCA1* BAC Tg mice (Figure 4-5B). However, this increase in cortical *ABCA1* mRNA is not necessarily accompanied by a proportional increase of ABCA1 protein. Compared to wild-type mice, animals with the *ABCA1* BAC alone or with the APP/PS1 transgenes alone each display a very modest but significant elevation of total ABCA1 protein in cortex ($p<0.01$, $N>10$, each group compared to wild-type controls) (Figure 4-5C). However, ABCA1/APP/PS1 mice display no further elevation in cortical ABCA1 protein levels, relative to APP/PS1 mice, despite having significantly increased human *ABCA1* mRNA levels ($p>0.05$, $N>10$) (Figure 4-5C). Cerebellar ABCA1 protein levels showed no significant difference among groups ($p>0.05$, $N>8$) (Figure 4-5C).

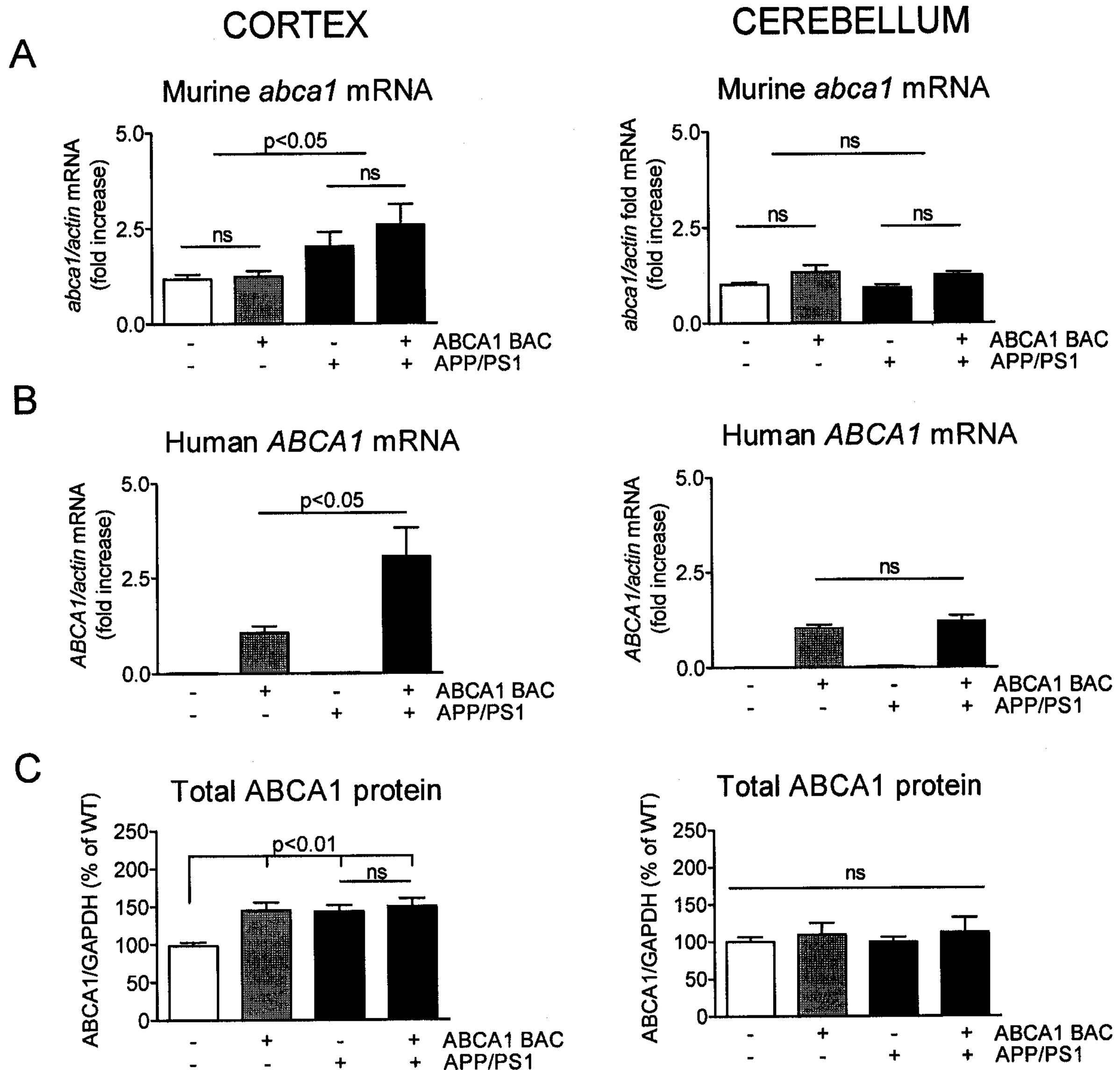

Figure 4-5: *ABCA1* mRNA is not induced until after 12 months of age.

Murine (A) and human (B) *ABCA1* mRNA levels were determined in cortex (amyloid rich) and cerebellum (amyloid poor) of wild-type, *ABCA1* BAC, APP/PS1 and ABCA1/APP/PS1 mice using QRT-PCR. Graphs are expressed as fold difference of all groups relative to wild-type controls, which were assigned an arbitrary value of one. Data represent at least duplicate measurements for 5-8 independent mice in each group. (C) ABCA1 protein levels were evaluated by Western blot and normalized to GAPDH as an internal loading control from N>8-11 independent mice per genotype for each group, analysed by one-way ANOVA. Data represent at least duplicate measurements per mouse.

4.3.5 ABCA1 expression is induced only in brain regions with advanced disease

To test whether endogenous murine ABCA1 expression was induced only in brain regions with very advanced amyloid deposition, we directly compared guanidine-extractable Aβ levels in cortex and cerebellum of 13-16 month old APP/PS1 mice, which did not contain the *ABCA1* BAC transgene. Cerebellum was examined because it was negative for any changes in ABCA1 expression in all of the experiments performed above even though the presence of the PS1ΔE9 mutation in the APP/PS1 model of AD is associated with Aβ and amyloid accumulation in the cerebellum. ThioS staining, however, reveals that this is not as extensive as cortical deposition (Figure 4-6A). Aβ40 levels were significantly higher in cortex than cerebellum ($p<0.0001$, $N>17$) (Figure 4-6B). Similarly, the cortex contained significantly more Aβ42 than cerebellum ($p<0.0001$, $N>17$) (Figure 4-6C). These data show that ABCA1 mRNA levels were elevated only in cortex, the brain region with highest Aβ levels. Our results are consistent with a model in which ABCA1 mRNA may be induced in response to advanced amyloid deposition, but where additional regulatory mechanisms may be prevent its increase at the protein level.

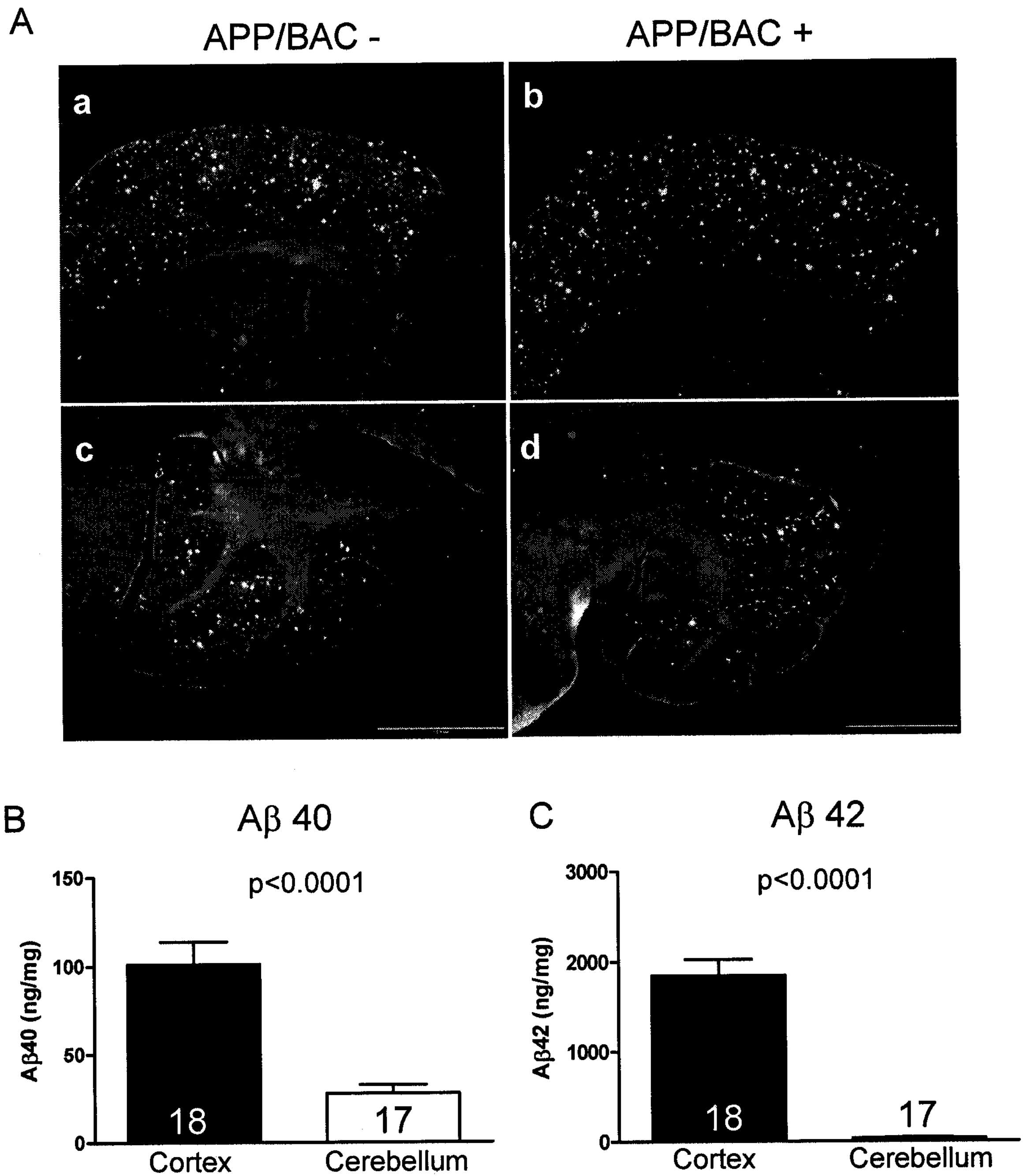

Figure 4-6: Overexpression of ABCA1 occurs in regions with high amyloid burden and Aβ levels:

(A) Thioflavine-S staining of hemicoronal cortical (a) and sagital cerebellar (c) section from a 13 month old APP/PS1 mouse. A cortical (b) and a cerebellar (d) section from an age- and sex matched ABCA1/APP/PS1 mouse id shown for comparison. Pictures correspond to 2.5x images for both cortex and cerebellum. Scale bars represent 1319 μm in (A). Guanidine-extractable Aβ40 (B) and Aβ42 (C) levels were determined by ELISA in cohorts of APP/PS1 mice. Student's t-test was used for statistical analysis

4.4 Discussion

ABCA1 is a critical regulator of apoE metabolism in the CNS [8, 9]. We and others have demonstrated that ABCA1 deficiency augments amyloidogenesis *in vivo* [19-21], suggesting the possibility that ABCA1 overexpression may inhibit amyloid formation. To address this question, we crossed human *ABCA1* BAC transgenic mice to the APP/PS1 model of AD and analyzed the impact of transgenic ABCA1 on amyloid deposition, Aβ levels, and apoE abundance. We specifically selected the *ABCA1* BAC transgenic model because it expresses human *ABCA1* in a physiologically accurate manner [22, 24] that has been shown to be atheroprotective *in vivo* [25, 26]. Furthermore, we confirmed that, in mice without amyloid deposits, *ABCA1* BAC transgenic mice exhibit a 50% increase in cortical ABCA1 protein and a 15% increase in cortical apoE protein compared to wild-type controls. These data demonstrate that BAC RP11-32H03 supports a modest but significant overexpression of ABCA1 in brain.

However, the presence of the *ABCA1* BAC made no significant impact on amyloid burden, Aβ levels, apoE abundance, or CSF cholesterol levels in mice with amyloid deposits, and raised the question of whether the BAC transgene remained expressed in mice with amyloid deposits. QRT-PCR experiments demonstrated that human *ABCA1* mRNA was clearly present in ABCA1/APP/PS1 and not in APP/PS1 cortex, as expected. However, in the amyloid-containing brain, BAC RP11-32H03 did not make any significant contribution to the total ABCA1 mRNA pool and no increase in ABCA1 protein levels was therefore detected. This is not due to failure of BAC transgene expression, however, because, in these same animals, human *ABCA1* constituted a significant proportion of total ABCA1 mRNA in liver and resulted in the expected increase in liver ABCA1 protein levels in ABCA1/APP/PS1 mice compared to APP/PS1 controls.

Further analysis revealed that ABCA1 expression appears to be responsive to local signals that may be generated in brain regions containing extensive amyloid deposits. Both human and murine ABCA1 mRNA levels were elevated in cortex but not in cerebellum of aged APP/PS1 mice, demonstrating regional specificity in the ABCA1 transcriptional response. However, this local induction of ABCA1 mRNA does not necessarily lead to increased protein levels, as ABCA1/APP/PS1 mice, which have the highest levels of ABCA1 mRNA, did not display a proportional increase in cortical ABCA1 protein. In these cohorts, a very subtle increase in cortical ABCA1 protein was observed in the presence of the BAC transgene alone, or in mice containing amyloid deposits, but these levels were not elevated additively in mice with the BAC as well as amyloid deposits. These observations suggest that ABCA1 may also be subject to yet undefined posttranscriptional regulatory mechanisms that may impose an upper limit on the degree to which physiologically regulated ABCA1 protein levels can be reached in brain regions that are prominently affected in AD.

Our observations also pose a potential challenge to the consideration of using selective *ABCA1* overexpression as a therapeutic means to increase apoE levels or lipidation in brains expected to develop AD neuropathology. Although we show that excess physiologically regulated ABCA1 can result in elevated brain apoE levels, this was only observed in animals without amyloid deposits. It remains to be determined whether driving selective ABCA1 expression to much higher levels in the brain, for example by using exogenous nonphysiological promoters, can override the regulatory mechanisms that appear to attenuate the expression of ABCA1 protein in the amyloid containing brain, and whether this leads to increases in apoE levels and lipidation that are sustained in the presence of AD neuropathology. Our study demonstrates that excess transgenic human ABCA1, expressed under the control of endogenous murine regulatory factors, is unable to mitigate AD neuropathology in mice. Of note, because the BAC RP11-32H03 used to generate this transgenic model is not as efficiently transcribed in brain as it is in liver, and because human transgenes may not be recognized as well in murine models

as they would be in humans, models based on increased murine *abca1* gene copies may constitute an alternative to evaluate the effect of physiologically regulated excees ABCA1 on AD neuropathology. Finally, this study also suggests for the first time that ABCA1 may be subject to transcriptional and posttranscriptional regulation during the pathogenesis of AD.

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Chapter 5 – Relevant contributions in co-authored papers

5.1 24(S)-Hydroxycholesterol participates in a liver X receptor-controlled pathway in astrocytes that regulates apolipoprotein E-mediated cholesterol efflux*.

In this paper, Abildayeva *et al* (Appendix 1) show that the brain-specific cholesterol metabolite produced by neurons, 24(*S*)-hydroxycholesterol, induces apoE transcription, protein synthesis, and secretion in a dose- and time-dependent manner in cells of astrocytic but not of neuronal origin. Moreover, 24(*S*)-hydroxycholesterol primes astrocytoma, but not neuroblastoma cells, to mediate cholesterol efflux to apoE [1]. This effect is likely mediated through an LXR-controlled pathway. Furthermore, apoE-mediated cholesterol efflux from astrocytic origin may be controlled by the ATP binding cassette transporters ABCA1 and ABCG1, as increase expression of both transporters was observed upon treatment with 24(*S*)-hydroxycholesterol. In conclusion, evidence is provided that 24(*S*)-hydroxycholesterol induces apoE-mediated efflux of cholesterol in astrocytes via an LXR-controlled pathway, which may be relevant for chronic and acute neurological diseases [1]. My contribution to this work consisted in the evaluation of the effects of 24(*S*)-hydroxycholesterol on the expression of both apoE and ABCA1 protein levels in primary murine glia cells (Figure 5.1).

Although the CNS meets ~95% of its cholesterol requirements by *de novo* synthesis, excess cholesterol cannot be degraded *in situ*. The main pathway for cholesterol elimination from the body is biliary secretion of cholesterol as bile acids or cholesterol [2]. The brain thus needs to secrete its excess sterol into the circulation for transport to the liver. Cholesterol is removed

* A version of this section has been published. Abildayeva K, Jansen PJ, Hirsch-Reinshagen V, Bloks VW, Bakker AH, Ramaekers FC, de Vente J, Groen AK, Wellington CL, Kuipers F, Mulder M. 24(S)-hydroxycholesterol participates in a liver X receptor-controlled pathway in astrocytes that regulates apolipoprotein E-mediated cholesterol efflux. J Biol Chem. 2006 May 5;281(18):12799-808.

from the brain predominantly in the form of 24(*S*)-hydroxycholesterol, a relatively polar and brain-specific cholesterol metabolite. 24(*S*)-hydroxycholesterol derives from the hydroxylation of cholesterol by the neuronal enzyme cholesterol 24(*S*)-hydroxylase (CYP46) [2]. 24(*S*)-hydroxycholesterol is also a naturally occurring LXR ligand [3], but its effects on genes involved in brain lipid metabolism had remained largely unexplored. In this study, Abildayeva *et al* tested the hypothesis that 24(S)-hydroxycholesterol represents a natural, brain-specific LXR ligand that regulates the expression of genes involved in apoE-dependent lipid trafficking within the CNS.

The authors had observed that 24(S)-hydroxycholesterol elevated apoE mRNA in astrocytoma, but not neuroblastoma cells. Furthermore, 24(S)-hydroxycholesterol was found to be a stronger inductor of intracellular apoE protein levels in astrocytoma cells than other natural LXR agonists, such as retinoic acid and free cholesterol. In addition to apoE, in astrocytoma cells, 24(S)-hydroxycholesterol induced the mRNA expression of ABCA1 and ABCG1, but not ABCG4. Finally, they had found that 24(S)-hydroxycholesterol enhanced apoE- and apoA-I-mediated cholesterol efflux from astrocytoma cells. Because all this observations related mainly to mRNA levels and had been done in cell lines of astrocytic origin, evaluation of cellular apoE and ABCA1 protein levels, as well as confirmation of the data in primary astrocytes was central to speculate about the potential relevance of these findings *in vivo*.

We evaluated the effects of 24(S)-hydroxycholesterol on apoE and ABCA1 protein levels in primary neonatal wild-type murine astrocytes. In our hands, 24(S)-hydroxycholesterol robustly increased ABCA1 protein levels two- to three-fold above vehicle treatment in primary astrocytes (Figure 5-1A, B). This induction was obtained with the smallest dose tested and did not significantly increase with higher concentrations of the oxysterol (Figure 5-1A, B). Cellular apoE levels, on the other hand, tend to increase with 24(S)-hydroxycholesterol treatment

(Figure 5-1A, C). High variability in cellular apoE protein levels was observed among independent experiments, and may explain why the results did not reach statistical significance. It also suggests that 24(S)-hydroxycholesterol is more effective at inducing expression of ABCA1 than apoE.

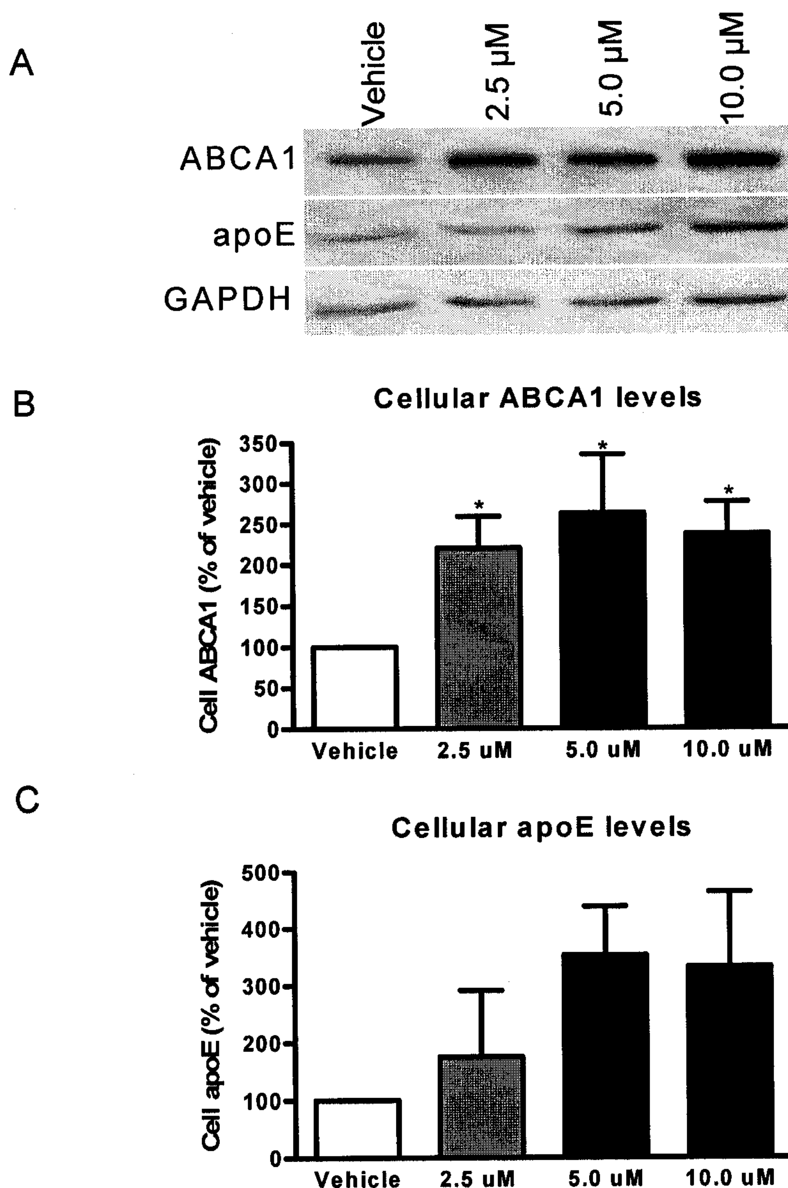

Figure 5-1: ABCA1 and apoE protein expression in response to 24(s) hydroxycholesterol treatment.

(A) ABCA1 (upper panel), apoE (middle panel) and GAPDH (lower panel) protein levels in whole cell lysates of primary mixed glial cultures were determined by Western blot and quantitated by densitometry. Intracellular GAPDH (lower panel) was used as an internal protein loading control. Graphs (B and C) are expressed as % of vehicle (cells treated with vehicle only were assigned a 100%) and illustrate four independent experiments, each measured in duplicate. Data represent the mean and standard deviation of cellular apoE (B) and ABCA1 (C) levels. One way ANOVA with Newman-Keuls post test was used to determine significant differences (* represents $p<0.01$ compared to vehicle only).

Together, the data presented in this manuscript shows that 24(*S*)-hydroxycholesterol is capable of inducing apoE and ABC transporter expression in astrocytes, and cholesterol efflux from these cells to exogenous apoE and apoA-I. These results thus suggest that release of 24(*S*)-hydroxycholesterol from neurons may prime astrocytes to secrete apoE-associated cholesterol. However, the physiological relevance of this pathway remains to be elucidated. Hydroxylation of cholesterol by neuronal CYP46 could occur either in response to overall excess brain cholesterol or to cell-specific neuronal increases in cholesterol content brought about by injury and neuronal degeneration. In the former case, secretion of cholesterol-containing particles by glia cells may serve to provide neurons with excess sterols for conversion to 24(*S*)-hydroxycholesterol and excretion from the CNS. Alternatively, neurons may require glial-derived cholesterol for repair and neuritic remodeling in the case of neuronal injury. It would thus be informative to evaluate whether cholesterol mass changes in astrocytes in response to 24(*S*)-hydroxycholesterol treatment. Glial cholesterol synthesis should be inhibited if generation of 24(*S*)-hydroxycholesterol responds primarily to overall brain sterol overload. In contrast, glial cholesterol synthesis should be activated if 24(*S*)-hydroxycholesterol primarily acts as a signal of neuronal cholesterol deficiency.

5.2 The cholesterol transporter *ABCG1* modulates the subcellular distribution and proteolytic processing of β-Amyloid Precursor Protein*

In this work, Tansley, Burguess *et al* (Appendix 2) show that overexpression of the cholesterol transporter ABCG1 results in altered trafficking and increased proteolytic processing of APP. ABCG1 has been shown to facilitate cholesterol efflux to HDL and is expressed in brain. Overexpression of ABCG1 in transfected HEK-APPSwe cells enhances secretion of Aβ peptides and sAPPα fragments in a manner that requires a functional ABCG1 transporter. The increase in Aβ generation is of special interest when one considers that the human ABCG1 gene maps to chromosome 21, and individuals with Down Syndrome (DS) typically manifest with AD neuropathology in their 30s. Consistent with the latter, ABCG1 mRNA levels are 2-fold more abundant in DS brain compared to age- and sex- matched normal controls. Finally, both Aβ and sAPPα levels are elevated in DS cortex relative to normal controls. These results suggest that ABCG1 increases the availability of APP as a secretase substrate for both the amyloidogenic and nonamyloidogenic pathways, and may contribute to the accelerated onset of AD neuropathology in DS. My contribution to this work consisted in the development of a Western blot protocol to detect and evaluate ABCG1 and APP protein levels in postmortem human brain samples to complement the mRNA data described above (Figure 5-2).

As described in Chapter 1, cellular cholesterol levels may modulate the processing of APP. Several reports suggest that high intracellular cholesterol levels results in enhanced release of

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* A version of this section has been published. Tansley GH, Burgess BL, Bryan MT, Su Y, Hirsch-Reinshagen V, Pearce J, Chan JY, Wilkinson A, Evans J, Naus KE, McIsaac S, Bromley K, Song W, Yang HC, Wang N, Demattos RB, Wellington CL. The cholesterol transporter ABCG1 modulates the subcellular distribution and proteolytic processing of β-amyloid precursor protein. J Lipid Res. 2007 Feb 10; [Epub ahead of print].

Aβ, whereas low intracellular cholesterol levels favor the non-amyloidogenic processing of APP [4]. ABCG1 is the founding member of the ABCG subclass of ABC transporters [5], and is highly expressed in murine brain [6, 7]. Biochemically, ABCG1 facilitates cholesterol efflux to HDL and redistributes intracellular cholesterol to plasma membrane domains that are accessible by cholesterol oxidase [8]. Intriguingly, the human *ABCG1* gene resides on chromosome 21 [9], suggesting that it maybe of interest for some of the clinical phenotypes associated with Down Syndrome (DS). One prominent phenotype in DS is the inevitable development of AD neuropathology by mid-late 30s [10]. Because ABCG1 modulates cellular cholesterol metabolism and may be overexpressed in DS, Tansley, Burguess *et al* hypothesized that ABCG1 modulates APP processing and contributes to the accelerated onset of AD neuropathology in DS.

The authors had observed that, in transfected HEK-APPSwe cells, overexpression of ABCG1 results in enhanced secretion of Aβ and production of sAPPα. This is likely due to increased proteolysis of APP by α-secretase and β-secretase in the presence of ABCG1. In addition, ABCG1 increases cell surface presentation of APP. To evaluate whether these effects are also present *in vivo*, Aβ and sAPPα levels were measured in postmortem DS cortex. Indeed, both Aβ and sAPPα levels were found to be elevated in DS cortex. Finally, to determine whether these effects on APP processing may be attributable to ABCG1 overexpression in DS subjects, quantitative RT-PCR was used to measure the levels of human ABCG1 mRNA in eight post-mortem trisomy 21 cases. ABCG1 mRNA levels in DS frontal cortex were two-fold more abundant than in control frontal cortex. In contrast to ABCG1, the authors had found that APP mRNA levels were not significantly increased in these same trisomy 21 cases relative to controls, although a clear trend toward increased APP expression was observed. Because protein levels do not necessarily correlate with mRNA levels, my contribution consisted in evaluating whether this increase in mRNA translated into increased ABCG1 protein levels.

Using Western blot analysis of total membrane preparations we observed that ABCG1 protein levels are 2-fold more abundant in DS frontal cortex compared to age-matched controls (p=0.008, N=4) (Figure 5-2). In contrast, APP protein levels were not significantly different in these same fractions (p=0.638, N=4) (Figure 5-2). Similar technology, which was based on a report by Albrecht *et al* [11], has also been successfully used to determine ABCA1 levels in human postmortem brain samples. To our knowledge, this constitutes the first report of protein detection of these two transporters in human brain.

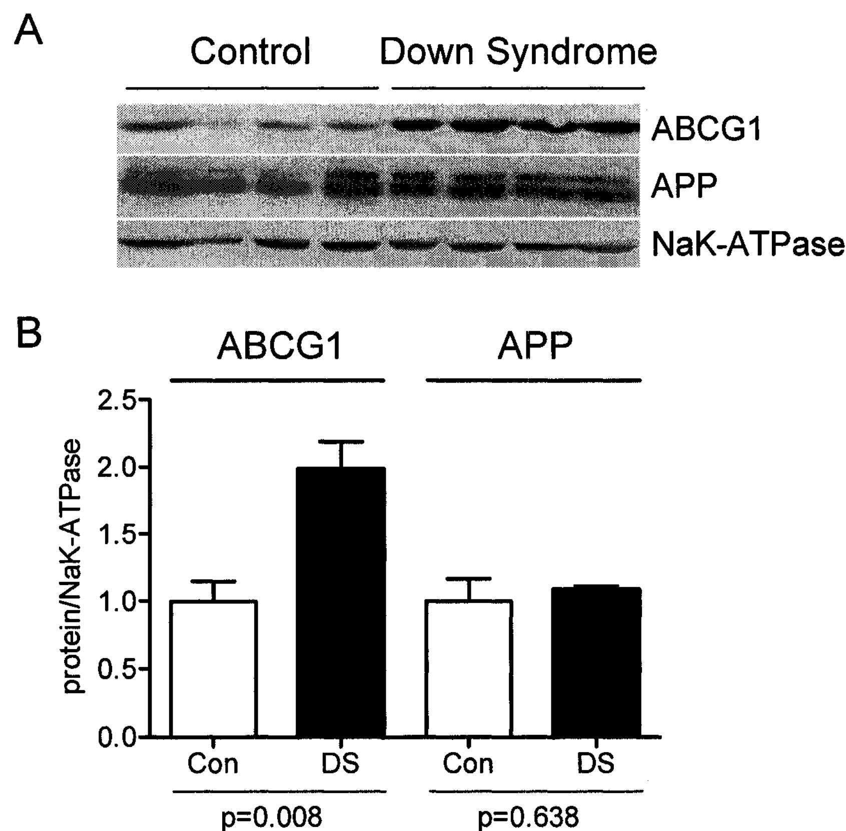

Figure 5-2: ABCG1 protein levels are significantly elevated in DS cortex.

(A) Western blots of total membrane fractions from four age-matched control and trisomy 21 cases. Blots were probed sequentially for ABCG1, APP, and NaK-ATPase that served as an internal loading control. (B) Relative ABCG1/NaK-ATPase and APP/NaK-ATPase values are shown, with the values in control fractions set to 1. Data represent the means and standard error of each case assayed in duplicate and analysed by Student's unpaired t-test.

Together, these data describe that transient overexpression of the cholesterol transporter ABCG1 affects the proteolytic processing and subcellular distribution of APP *in vitro*. In cultured HEK-APPswe cells, expression of functional ABCG1 elevates Aβ and sAPPα secretion and increases the proportion of APP that is present at the cell surface. The observation that DS cortex contains 2-fold more ABCG1 mRNA and protein than age-matched control samples and exhibits increased Aβ and sAPPα levels are consistent with the above *in vitro* observations. In sum, these data support the hypothesis that excess ABCG1 in DS may result in an altered distribution of APP, facilitating the generation of neurotoxic Aβ species and accelerating the onset of AD neuropathology. However, the results of human postmortem brains need to be evaluated cautiously. Firstly, the results are correlative and do not rule out the possibility that other chromosome 21 genes in addition to ABCG1 may also play a role in the development of AD neuropathology in DS subjects. In addition, increased number of samples and independent samples from both affected and unaffected brain regions need to be assessed for both APP and ABCG1 expression. Finally, future studies will be required to evaluate the impact of selective overexpression or deficiency of ABCG1 on AD.

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Chapter 6 – Conclusion

6.1 ABCA1 and brain lipoprotein metabolism

The recognition of ABCA1 as the defective gene in Tangier Disease revolutionized our understanding of HDL metabolism. It is currently well established that ABCA1 is a key component in the biogenesis of circulating plasma HDL particles. What remained unclear, however, was the relevance of ABCA1 expression in the brain, since CNS lipoprotein metabolism is largely independent of the periphery. Indeed, the question whether ABCA1 was involved in the generation of the HDL-like lipoproteins found in CSF remained largely unanswered. In this work, novel evidence is provided suggesting a crucial role for ABCA1 in regulating CNS lipoprotein metabolism.

In chapter two I show, based on the effects of ABCA1 absence, that ABCA1 is required to maintain normal brain apoE levels and lipidation. Specifically, the absence of ABCA1 in mice results in approximately 80% reduction in brain apoE levels. This dramatic effect suggests that few compensatory pathways are able to balance the impact of ABCA1 absence on CNS apoE metabolism. The exact mechanisms underlying this reduction have yet to be completely elucidated; however, we have provided initial data suggesting that ABCA1 is involved in both secretion and lipidation of apoE. Poor lipidation of apoA-I has been shown to increase renal catabolism of this apolipoprotein [1]. This would suggest that, in the absence of ABCA1, reduction of apoE secretion and increase of apoE degradation may be responsible for the decreased apoE levels observed in ABCA1-deficient brains.

Of note, although apoE and apoA-I, the two main CSF apolipoproteins, are dramatically reduced in ABCA1-deficient mice, CSF cholesterol level is only decreased by 20% in the absence of ABCA1 compared to wild-type controls. This suggests that, unlike CNS apoE and

circulating plasma HDL levels, several ABCA1-independent mechanisms are able to compensate for the lipid trafficking disturbances that occur with the lack of ABCA1. ApoJ levels are not altered in the absence of ABCA1, making this apolipoprotein an unlikely candidate to play an important compensatory role. ApoD, on the other hand, has been suggested to balance the absence of apoE and apoA-I in peripheral nerve regeneration [2]. ApoD expression is increased 20- to 50-fold in the CNS of apoE-deficient animals [3], possibly explaining the mild brain and behavioral phenotype of these animals. ApoD overexpression in combination with other currently unknown compensatory pathways may be active in ABCA1-deficient animals, and merits further exploration.

No studies have been yet published on apoE levels in the CSF of TD patients. However, plasma apoE levels have been reported to be reduced by ~50% in TD patients compared to controls [4, 5], a feature that ABCA1-deficient animals replicate [6, 7]. It would be thus highly interesting to evaluate the incidence and neuropathological features of AD in TD patients. To our knowledge, there is only one case report of a proband with a compound mutation in the *ABCA1* gene who developed and died of complications related to cerebral amyloid angiopathy (CAA) [8].

In contrast to the dramatic effect of ABCA1-deficiency on apoE levels, a moderate increase in ABCA1 expression results in a mild elevation in apoE levels in murine brain homogenates, but not in CSF, as described in chapter four. This result suggests that, under physiological conditions, a modest overexpression of ABCA1 is not sufficient to robustly increase brain apoE levels and that elevation of apoE protein levels may be modulated by a variety of ABCA1-independent mechanisms. In summary, our work provides one of the first indications that the absence of ABCA1 profoundly affects brain lipid metabolism *in vivo.* Further studies are needed to address whether more robust overexpression of ABCA1 is sufficient to drive

significant increases in apoE levels or lipidation, thus opposing the effects of ABCA1-deficiency.

Our results suggest that, at least in mice, ABCA1 is the main determinant of apoE lipidation. In addition, and as suggested by this thesis work, apoE lipidation status significantly impacts the ability of apoE to modulate amyloid deposits. As per our observations, it is possible to hypothesize that poor lipidation of apoE renders it proamyloidogenic. This would suggest that, in humans, isoform-specific differences in apoE lipid content may partially explain the association between apoE isoform and amyloid burden. More specifically, given that expression of apoE4 is associated with higher amyloid burden than apoE3 expression, one would expect, based on our findings, that apoE4 is less lipidated than apoE3. In accordance with this, apoE3-expressing astrocytes release approximately 2.5-fold more cholesterol into the medium than apoE4-expressing astrocytes. No differences were observed between genotypes in either levels of secreted apoE or lipoprotein particle size [9]. This suggests that the cholesterol/apoE ratio is higher in apoE3 than in apoE4 particles. Isoform-specific association of apoE with different lipoprotein classes has been robustly demonstrated in human plasma. ApoE3 and apoE2 preferentially bind to HDLs, whereas apoE4 is preferentially found on triglyceride-rich VLDLs [10]. Studies analyzing CSF cholesterol levels in relation to apoE phenotype have yielded mixed results [11, 12]. Fagan *et al.* did not find any differences in CSF cholesterol levels in healthy elderly by apoE genotype [11]. Yamauchi *et al.*, on the other hand, report that apoE4 carriers have significantly lower CSF cholesterol levels than apoE3 carriers [12]. Of note, the samples used in the latter study included CSF from healthy individuals and patients with neurological diseases, which may confound the results. Further studies in fasted AD patients and healthy age- and sex- matched controls are required to settle these discrepancies.

Wahrle *et al* reported that ABCA1-deficient glia secrete lipid-poor apoE particles [7], suggesting that ABCA1 plays an important role in apoE lipidation. ApoE2 has been shown to induce higher cholesterol efflux than apoE3 and apoE4 from cultured neurons and glia [13]. We reported a significant decrease in cholesterol efflux to both apoA-I and apoE in primary ABCA1-deficient glia compared to wild-type controls. This result corroborates that our lipoprotein preparations were functional, and able to induce ABCA1-specific cholesterol efflux. However, unlike Michikawa *et al.*, we did not detect an isoform-specific effect on cholesterol efflux to apoE in wild-type cells. Since our apoE preparations derived from GST-fusion proteins expressed in bacteria, and required a stringent purification step before use, it is possible to speculate that our apoE acceptors may have retained a general ability to interact with ABCA1 and induce cholesterol efflux, but may have lost some isoform-specific properties during the purification step. Supporting this hypothesis are observations that suggest that peptides with amphipathic helices induce ABCA1-mediated lipid efflux [14] . Whether the isoform-specific cholesterol efflux reported by Michikawa *et al* is ABCA1-dependent or not, remains to be elucidated. Ideally, astrocyte-derived conditioned media containing each of the isoforms should be used to test this hypothesis, as it corresponds to a more biologically relevant lipoprotein preparation, both in the conformation and lipidation of the secreted apolipoproteins, than recombinant apolipoproteins.

6.2 ABCA1 and Alzheimer neuropathology

The observation that the absence of ABCA1 dramatically reduces brain apoE levels led us to hypothesize that ABCA1 deficiency would result in decreased amyloid formation in murine models of AD, given that amyloid formation is positively associated with apoE levels. Surprisingly, lack of ABCA1 and the consequent reduction in apoE levels resulted in at least as much amyloid deposition as in the presence of wild-type levels of apoE, without affecting Aβ generation. This result suggests for the first time that reduction of apoE lipidation greatly affects

apoE function *in vivo*. Moreover, it suggests that either increased deposition or decreased clearance of Aβ peptides occurs in the absence of ABCA1.

Although ABCA1-deficiency greatly affects amyloid metabolism *in vivo*, increased ABCA1 gene-dose did not have the expected protective effect in the studies we have completed to date. In fact, expression of human ABCA1 in *ABCA1* BAC transgenic mice resulted in only a very subtle increase in ABCA1 expression specifically in the absence of the APP/PS1 transgenes. Furthermore, brain, but not hepatic, ABCA1 overexpression was abolished in the presence these AD-related transgenes, suggesting that AD neuropathology may be modulating brain ABCA1 expression. These observations suggest that the *ABCA1* BAC transgenic model of ABCA1 overexpression is less than ideal to investigate whether overexpression of ABCA1 can mitigate AD neuropathology *in vivo*. It thus remains to be determined whether driving ABCA1 expression to higher levels impacts amyloid formation *in vivo*.

Behaviorally, the question of whether ABCA1-deficiency *per se*, or in combination with AD neuropathology induces cognitive dysfunction, remains unanswered. Although peripheral neuropathy is found in ~ 50% of TD patients [15], no overt CNS neuropathology or behavioral phenotype has been described either in TD subjects or in ABCA1-deficient animals. Cognitive and neuropathological studies performed on apoE-deficient mice have resulted in mixed reports, suggesting that complete absence of brain apoE does not result in robust cognitive impairment. Cognitive function of PDAPP transgenic AD mice in the absence and presence of endogenous apoE has been evaluated in two studies with mixed results [16, 17]. Dodart *et al* showed that deficiency of apoE exacerbates an object recognition impairment present in PDAPP mice that express apoE [17]. In contrast, Nilsson *et al* reported that spatial working memory dysfunction is greater in apoE-expressing PDAPP mice compared to apoE-deficient PDAPP controls [16]. The authors suggest that the differences observed in both studies may be due to different sensitivities of each aspect of cognition (identification versus working memory)

to different forms of Aβ deposition. So, they propose that object recognition has a weak correlation with Thioflavin-S positive Aβ deposits [17], whereas working memory, as measured by Nilsson *et al*, is affected by both diffuse and compact parenchymal Aβ deposits [18].

In agreement with Dodart's study, however, a second AD transgenic animal model, in which Aβ deposition is mainly of diffuse nature, presents with significant memory dysfunction, suggesting that soluble Aβ may be the main determinant of cognitive impairment in AD [19]. Accordingly, it has been suggested that Aβ oligomers rather thant Aβ fibrils constitute the most toxic species of Aβ [20]. Together, these data suggest that amyloid may be a protective mechanism by which toxic Aβ species are deposited and neutralized. Because the absence of ABCA1 results in increased amyloidogenesis when corrected for total apoE levels, it would be of interest to evaluate whether increased levels of poorly lipidated apoE in the brains of transgenic AD models indeed results in a shift of soluble Aβ to fibrillar deposits and improvement of cognitive dysfunction. Finally, because amyloid formation has been shown to be apoE-isoform dependent in AD transgenic animals expressing human apoE, it would be of interest to evaluate whether ABCA1-deficiency affects amyloid deposition in an isoform-dependent manner.

6.3 Other ABC transporters in brain lipid metabolism.

Of the seven ABC transporter subfamilies (A to G), members of both ABCA and ABCG subfamilies have been linked to lipid metabolism, are expressed in brain, and may thus influence the outcome of AD and other neurodegenerative diseases. In addition to ABCA1, other ABCA subfamily members may play important roles in CNS lipid trafficking. For example, ABCA2 is ubiquitous, but its expression is highest in brain [21]. Little is known about the specific function of ABCA2. Sterol-sensitive transcriptional regulation of this transporter has been demonstrated *in vitro* [22, 23] and ABCA2 has also been implicated in multidrug resistance in

transfected HEK cells [24]. Of note, ABCA2 has been implicated in AD as a genetic risk factor and as a modulator of APP metabolism. Overexpression of ABCA2 in stably transfected APP (695) 293 cells results in increased cellular APP protein levels and Aβ secretion [25]. Furthermore, genetic analyses have suggested that a synonymous single nucleotide polymorphism may be associated with early-onset AD [26, 27]. Very recently, a 'skittish' phenotype has been described for ABCA2-defcient mice [28]. These animals present with tremor, hyperactivity and reduced body weight. At the histological level, absence of ABCA2 resulted in increased myelin sheath thickness and decreased myelin membrane periodicity (compaction) in knockout compared to wild-type spinal cord. Of interest, hyperactivity and reduction in body weight are more marked in females, suggesting a role for ABCA2 in hormone-dependent neurological and/or developmental pathways. These observations suggest that ABCA2 may have an important role in brain lipid homeostasis and AD, but further studies are needed to confirm and explain this observation.

ABCA3 is mainly expressed in lung, brain and heart. The highest expression of ABCA3 is found in lung [29, 30], where it localizes to lamellar bodies of alveolar type II pneumocytes [31, 32]. Lamellar bodies contain pulmonary surfactant and mutations in ABCA3 are associated with fatal neonatal pulmonary surfactant deficiency [33]. However, the exact function of ABCA3 in the metabolism of pulmonary surfactant remains to be elucidated. Moreover, although ABCA3 and ABCA2 have been shown to be highly expressed in primary human oligodendrocytes [34] and during brain development [35], their role in brain lipid homeostasis remains unknown.

ABCA7 has been shown to be predominantly expressed in myelolymphatic tissues and brain [35, 36]. Initially thought to mediate the generation of HDL similarly to ABCA1, *in vivo* studies suggest a modest effect of ABCA7-deficiency on plasma HDL levels [37] . In addition, ABCA7 does not rescue the plasma HDL deficiency caused by the defect of ABCA1 function in Tangier Disease patients or mice. Together, these data suggest a specific function for ABCA7 in lipid

metabolism that is unrelated to ABCA1. Indeed, ABCA7, but not ABCA1, has recently been implicated in phagocytosis of apoptotic bodies by macrophages. ABCA7 heterozygous alveolar macrophages exhibit a significantly decreased uptake of apoptotic cells *in vivo* when compared to wild-type controls [38]. This is of potential interest to AD, as microglia have been shown to phagocyte fibrillar Aβ deposits [39], possibly constituting an Aβ clearing mechanism.

Finally, emerging evidence suggests that ABCA6, and its related transporters ABCA9 and ABCA10, are expressed in the CNS and respond to cellular cholesterol levels [40-42], suggesting a potential role for these proteins in brain sterol metabolism.

Of the ABCG subfamily of transporters ABCG1 and ABCG4 have been described in brain and are consistently implicated in lipid trafficking. Both half transporters are highly expressed in brain [35, 43], and are reported to mediate cholesterol efflux to HDL [44-47]. Furthermore, ABCG1 has been directly implicated in cerebellar glia cholesterol metabolism [48]. Together, this suggests that an intricate network of lipid transporters may be involved in maintaining lipid homeostasis in the CNS, however, many details still remain to be fully understood.

6.4 Aβ peptides as regulators of lipid metabolism

Many studies have focused on the effects of cellular cholesterol modulation on Aβ production. However, some studies also suggest that Aβ may reciprocally affect cholesterol metabolism. Sphingomyelinases mediate the hydrolysis of sphingomyelin into ceramide and phosphocholine. Grimm *et al.* showed that Aβ42 directly activates neutral sphingomyelinase (SMase) activity, resulting in reduced sphingomyelin levels. Aβ40, on the other hand, inhibits the activity of hydroxymethylglutaryl-CoA reductase (HMCoAR) and thus results in decreased de novo synthesis of cholesterol [49]. Consistent with the action of Aβ42 on SMases, ceramide

levels are significantly increased in vulnerable brain regions of AD patients, compared to normal controls subjects [50, 51]. Of interest to our work is the observation that treatment of CHO cells with ceramide results in a 2-3-fold increase in ABCA1-dependent cholesterol efflux to apoA-I, and in significantly elevated plasma membrane levels of ABCA1 protein [52]. This would suggest that increased ceramide levels in AD brains may result in elevated brain ABCA1 protein levels or cholesterol efflux. However, whether ABCA1 protein levels are indeed altered in vulnerable AD brain regions in humans remains to be elucidated.

Aβ oligomers have also been reported to induce cholesterol and phosphocholine efflux from primary neurons and astrocytes in a time- and dose- dependent manner [53]. The resulting particles exhibit HDL-like density. Of note, neuronal-derived Aβ-lipid complexes were devoid of apoE, whereas astrocyte-derived particles exhibited apoE immunoreactivity [53]. However, the exact mechanism underlying the induction of Aβ - dependent lipid efflux from these cells remains unknown. In particular, studies addressing whether lipid efflux occurs through passive diffusion or is actively mediated by a transporter protein are required. Experiments addressing Aβ-induced cholesterol efflux in primary CNS-derived cells obtained from animals deficient in ABCA and ABCG transporters would be of particular interest.

6.5 Brain lipid metabolism as therapeutic target in AD

LXR agonists regulate the transcription of a variety of genes that regulate lipid homeostasis, including ABCA1 and apoE [54]. Recently, two publications have consistently shown a decrease in Aβ generation in transgenic murine models of AD treated with TO-901317, an LXR agonist [55, 56], suggesting that treatment with LXR agonists may be of therapeutic benefit in AD. Use of LXR agonists is associated with an increase in ABCA1 protein and increased cholesterol efflux in CNS-derived cells [57, 58]. Of interest, ABCA1-deficient mice treated with TO-901317 did not exhibit decreased Aβ secretion, suggesting that ABCA1 may be a key mediator of the

beneficial effects of LXR treatment (Wahrle *et al*, unpublished communication). LXR agonists are currently under intense investigation, as they constitute promising therapeutic targets for the treatment of atherosclerosis [54]. However, early synthetic LXR-selective agonists such as TO901317 (Tularik) induce significant hypertriglyceridemia and hepatic steatosis due to the activation of genes involved in fatty acid biosynthesis and the resulting accumulation of triglycerides [59], an undesired side effect that needs to be overcome before these compounds are considered for therapeutic purposes. One strategy to achieve this goal is to identify LXR agonists with selective activity for specific LXR target genes. The feasibility of this strategy is exemplified by two recent reports describing differential and selective activity of two synthetic LXR agonists on different genes involved in lipid metabolism [60, 61]. A full characterization of the impact of these compounds on the expression of genes involved in lipid metabolism in the CNS is a logical second step to understand the potential therapeutic benefit of LXR agonists in AD.

Treatment with statins, another drug class that targets lipid metabolism by decreasing de novo synthesis of cholesterol, was initially reported as protective against AD [62, 63]. These initial observations have been met with controversial results (reviewed in [64]). Although a therapeutic role of statins in AD still needs confirmation, it is worthwhile noting that several publications suggest that the mechanism by which statins reduce Aβ production could be pleitropic, and do not necessarily depend on significantly reducing tissue cholesterol levels (reviewed in [65]). Whether these pathways include modulation of ABCA1 expression and lipid efflux remains unclear. Studies addressing the impact of statins on ABCA1 expression and apoA-I-induced cholesterol efflux in cultured macrophages have reported conflicting results [66-70].

Together, these studies suggest that compounds that impact lipid metabolism may be of therapeutic use in AD. However, more detailed mechanistic insight into potential modes of action of these and similar drugs is needed. More specifically, the role of ABCA1 and other lipid transporters as effectors of drugs that target lipid metabolism needs to be carefully assessed.

6.6 Future Directions

Several lines of future investigation are suggested by this work. Firstly, it will be of interest to extend our observations of ABCA1-deficiency on AD neuropathology to a behavioral correlate. Analysis of cognitive function of ABCA1-deficient animals both in the presence and absence of AD-inducing transgenes should provide insights into this question. Examination of general sensorimotor function and reflexes, as well as evaluation of specific cognitive faculties, such as working memory, should be included. The presence of neuropathy in Tangier patients suggests that motor function and/or nociceptive reflexes may be altered in ABCA1-defcient animals. The absence of overt brain dysfunction in TD subjects in addition to the mild phenotype of apoE-deficient animals suggests that no overt behavioral deficits are expected in this animal model. Similar to apoE-deficient animals, where strong compensatory mechanisms may be at play, experimental paradigms that include brain injury may be necessary to uncover behavioral phenotypes that are ABCA1-dependent. Hippocampal lesion models with a clear behavioral phenotype may be thus useful to reveal the impact of ABCA1-deficiency on neuronal plasticity in the CNS, and should be prioritized over studies that look at baseline behavior of ABCA1-deficient rodents.

Based on our observation that amyloid burden is elevated in ABCA1-deficient animals, increased memory deficits may be observed in these mice. Memory tests that are sensitive to hipppocampal amyloid deposition, like the one used by Nilsson *et al* [16], will be of particular interest to use in young and aged animals. In addition, a thorough neuropathological analysis of the brains of these mice would complement our findings on amyloid burden by evaluating the detailed morphology of neuritic plaques, including dystrophic neurites and reactive gliosis, as they may differ by ABCA1 genotype. This is of special importance if one considers that amyloid composition may differ in the absence of ABCA1, as we observed that plaque-associated apoE levels were higher in ABCA1-deficient animals compared to wild-type controls.

The observation that ABCA1-deficiency, with its dramatic reduction in both apoE and apoA-I levels, only moderately reduces CSF cholesterol levels [6, 7], raises the question of what compensatory mechanisms may be active in the CNS. This is of special interest when one compares the 70% reduction in plasma cholesterol levels observed in mice lacking ABCA1 [71] with the 24% reduction in CSF cholesterol in the same animals [7]. This would suggest that CNS-specific compensatory mechanisms may be at play that are not active or absent in the periphery. Expression analysis of other lipoprotein-related genes in the brains of ABCA1-deficient animals would constitute a suitable approach to address this issue. Likely candidates to counteract the lipid efflux impairments brought about by the absence of ABCA1 include other ABCA and ABCG transporters. To balance the partial deficiency of apoE, other CNS-synthesized apolipoproteins, such as apoD, may be upregulated. As well, downregulation of lipoprotein receptors may occur in response to increased cellular cholesterol levels.

A further question raised by our results in total ABCA1-deficient animals relates to the cell-specific contribution of ABCA1 to brain lipid metabolism. Our observation that ABCA1 is the main determinant of apoE lipidation in murine brain provides an excellent opportunity to elucidate what cell type within the CNS is primarily responsible for the generation of the HDL-like particles found in the CSF. ABCA1 expression has been described in all brain cell types, suggesting that any of these could participate in lipidation of apoE and generation of the CSF HDL-like particles. Outside the brain, hepatic and intestinal ABCA1 have been demonstrated to contribute approximately 70% and 30% of HDL biogenesis respectively [1, 72]. Interestingly, these two organs also constitute the sites of apoA-I synthesis in the body. It is thus tempting to draw parallels between CNS and peripheral lipoprotein metabolism, and hypothesize that glia cells may constitute the primary source of apoE lipids, as the existing evidence suggests that CNS apoE is mainly of glial origin. A murine model expressing an astrocyte-specific deletion of ABCA1 would constitute a valuable tool to initiate the evaluation of this question *in vivo*.

A further avenue of study relates to the question of whether ABCA1 overexpression results in elevated apoE levels and lipidation in the CNS, the opposite effects of ABCA1-deficiency. For this, a model with robust, autonomous overexpression of CNS ABCA1 is needed. Because LXR agonists have shown potential as Aβ-reducing agents [55, 56], and some of its effects may be mediated by ABCA1 overexpression (Wahrle, unpublished observation), it would be of special interest to analyze whether APP processing is affected in ABCA1 overexpressing animals. This would establish whether the Aβ-reducing effects of LXR treatment are at least partially mediated specifically by ABCA1. However, if one extrapolates the results of the ABCA1-deficient model, where no overt changes in Aβ generation were observed in comparison with ABCA1-expressing controls, it seems unlikely that ABCA1 overexpression may affect APP processing. It is more likely that ABCA1 overexpression impacts AD neuropathology by influencing the metabolism of apoE, rather than altering the rate of Aβ generation.

Finally, the question of whether the impact of ABCA1 deficiency observed in our murine models also applies to humans remains unclear. Because ABCA1-deficient mice recapitulate various aspects of TD [71], including the reduction in plasmatic apoE levels [6, 7], it is reasonable to hypothesize that lack of ABCA1 may have similar effects on brain apoE metabolism in ABCA1-deficient humans and mice. However, whether our observations of increased amyloid deposition in the absence of ABCA1 also hold true for humans is not clear. The biggest caveat in extrapolating our results in rodents to humans is the fact that our AD models only replicate a subset of the neuropathological hallmarks of AD [73]. Furthermore, recent evidence suggests that the spatial progression of amyloid deposition in familial forms of AD may be different from that observed in sporadic cases (Klunk WE, unpublished observation). This suggests that the physiopathology of familial AD may differ from the sporadic form. This in turn suggests that factors that influence the former, such as ABCA1-deficiency and poor lipidation of apoE, may not affect the latter.

Nevertheless, it would be highly informative to evaluate both incidence and neuropathological features of AD, as well as CSF apoE levels and apolipoprotein composition in TD patients and/or subjects with FHA. The majority of mutations in *ABCA1* are private mutations occurring in individual families and have a broad range of phenotypes and biochemical effects. For example, HDL cholesterol levels in patients carrying heterozygous *ABCA1* mutations range from 30% to 100%, instead of the expected 50% due to loss of one *ABCA1* allele [74]. This suggests that different mutations may affect ABCA1 function in specific manners, some allowing residual ABCA1 activity, while others may act as dominant negative mutations. It would be therefore imperative to correlate both AD neuropathology and CSF apolipoprotein and lipid composition with the specific phenotype and biochemical activity of each individual *ABCA1* mutation studied.

6.7 Summary

This thesis constitutes an original contribution to our understanding of the role of ABCA1 in brain lipid metabolism. ABCA1 had been widely recognized as a key protein in the generation of peripherally circulating HDL particles. The studies described herein complement this model, and suggest that ABCA1 is also essential for CNS lipoprotein metabolism. They show, for the first time, that ABCA1 is the main determinant of lipidation and levels of apoE in murine brain, and that lipidation status of apoE has dramatic functional repercussions *in vivo.* Furthermore, through its effects on brain apoE homeostasis, ABCA1 may also affect the development of AD *in vivo.* In sum, the main hypothesis driving my thesis work, that ABCA1 plays a key role in the development of AD neuropathology and in brain lipid metabolism, has been answered affirmatively, and constitutes the initial step in elucidating the physiological, and possibly therapeutic, functions of CNS ABCA1 in health and disease.

6.8 Reference list

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Appendix 1: Co-authored Papers

Appendix 1.1: 24(S)-hydroxycholesterol participates in a liver X receptor-controlled pathway in astrocytes that regulates apolipoprotein E-mediated cholesterol efflux.

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24(*S*)-Hydroxycholesterol Participates in a Liver X Receptor-controlled Pathway in Astrocytes That Regulates Apolipoprotein E-mediated Cholesterol Efflux*

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Both apolipoprotein E (apoE) and 24(*S*)-hydroxycholesterol are involved in the pathogenesis of Alzheimer disease (AD). It has been hypothesized that apoE affects AD development via isoform-specific effects on lipid trafficking between astrocytes and neurons. However, the regulation of the cholesterol supply of neurons via apoE-containing high density lipoproteins remains to be clarified. We show for the first time that the brain-specific metabolite of cholesterol produced by neurons, *i.e.* 24(*S*)-hydroxycholesterol, induces apoE transcription, protein synthesis, and secretion in a dose- and time-dependent manner in cells of astrocytic but not of neuronal origin. Moreover, 24(*S*)-hydroxycholesterol primes astrocytoma, but not neuroblastoma cells, to mediate cholesterol efflux to apoE. Similar results were obtained using the synthetic liver X receptor (LXR) agonist GW683965A, suggesting involvement of an LXR-controlled signaling pathway. A 10–20-fold higher basal LXRα and -β expression level in astrocytoma compared with neuroblastoma cells may underlie these differential effects. Furthermore, apoE-mediated cholesterol efflux from astrocytoma cells may be controlled by the ATP binding cassette transporters ABCA1 and ABCG1, since their expression was also up-regulated by both compounds. In contrast, ABCG4 seems not to be involved, because its expression was induced only in neuronal cells. The expression of sterol regulatory element-binding protein (SREBP-2), low density lipoprotein receptor, 3-hydroxy-3-methylglutaryl-CoA reductase, and SREBP-1c was transiently up-regulated by GW683965A in astrocytes but down-regulated by 24(*S*)-hydroxycholesterol, suggesting that cholesterol efflux and synthesis are regulated independently. In conclusion, evidence is provided that 24(*S*)-hydroxycholesterol induces apoE-mediated efflux of cholesterol in astrocytes via an LXR-controlled pathway, which may be relevant for chronic and acute neurological diseases.

Disturbances in brain cholesterol homeostasis are associated with the onset of severe neurological diseases (1) and have recently been suggested to play a key role in the development of Alzheimer disease (AD)[4] (2, 3). The brain, although composing just 2% of the total body mass, contains about a quarter of an individual's whole body unesterified cholesterol. Brain cholesterol originates almost exclusively from *in situ* neosynthesis (1); circulating cholesterol is prevented from entering the brain by the blood-brain-barrier (4). Because cholesterol cannot be degraded and is neurotoxic at high levels, excess cholesterol is secreted from the brain into the circulation (5). Cholesterol is removed from the brain predominantly (about 60%) in the form of the relatively polar brain-specific metabolite, 24(*S*)-hydroxycholesterol, formed by the enzyme cholesterol 24(*S*)-hydroxylase (CYP46) (1). The remaining 40% of cholesterol is secreted from the brain via an unknown pathway that may involve apoE (6). CYP46 is expressed predominantly by neurons (7, 8). Several studies have suggested a role for 24(*S*)-hydroxycholesterol in the pathogenesis of AD (9–11). Polymorphisms of CYP46 have been linked to AD, and the expression of this enzyme appeared to be shifted from neurons to glia in AD patients (12). Finally, increased levels of 24(*S*)-hydroxycholesterol levels have been detected in cerebrospinal fluid of AD patients (13).

24(*S*)-Hydroxycholesterol is a natural ligand of the liver X receptors (LXR), which have recently been identified as central players in the regulation of cholesterol metabolism (14, 15). LXR belong to the nuclear hormone receptor superfamily, and two isoforms, *i.e.* LXRα and LXRβ, have been identified that are activated by oxysterols. Both isoforms of LXR are expressed in the central nervous system (16) and are thought to be involved in the regulation of brain cholesterol metabolism. LXRα/β-null mice show a variety of central nervous system defects upon aging, including lipid accumulation, astrocyte proliferation, and disorganized myelin sheaths (17). The synthetic LXR ligand T0901317 was found to induce the expression of apoE and of the ATP binding cassette transporters A1 (ABCA1) and G1 (ABCG1) in astrocytes (18). However, reported *in vivo* effects of T0901317 on apoE expression in mouse brain are inconsistent (16, 19, 20).

The strongest genetic risk factor known for sporadic AD is apolipoprotein E4 (apoE4), one of the three common apoE variants (apoE2,

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[4] The abbreviations used are: AD, Alzheimer disease; HMG, hydroxymethylglutaryl; LDLR, low density lipoprotein receptor; ABC, ATP binding cassette; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; QRT-PCR, real-time quantitative PCR; LXR, liver X receptor.

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apoE3, apoE4) in humans (21, 22). ApoE is a key player in the transport of cholesterol in the circulation (23) and is also thought to fulfill such a role within the brain (24). Astrocytes are the predominant source of apoE in the brain. These cells secrete apoE in association with cholesterol and phospholipids in the form of small, high density lipoprotein-like particles (25). It has been suggested that these particles provide neurons with cholesterol required for the formation of new membranes, *e.g.* during development, regeneration after injury, or during the formation of new synaptic contacts (26). It has been hypothesized that apoE may affect the pathogenesis of AD by isoform-specific effects on lipid trafficking between astrocytes and neurons (27). Indeed, apoE in combination with cholesterol induces the outgrowth of neurites in an isoform-specific manner in neuronal cultures (28). However, factors that regulate the supply of glial-derived apoE-containing lipoproteins are poorly understood.

In this study we tested the hypothesis that 24(*S*)-hydroxycholesterol represents a natural brain-specific LXR ligand that is involved in the regulation of the apoE-mediated lipid supply. For this purpose the effects of 24(*S*)-hydroxycholesterol and the synthetic LXR agonist GW683965A on the expression of apoE and additional LXR target genes involved in cholesterol efflux were compared using human neuroblastoma and astrocytoma cell lines as well as primary astrocytes. We found that 24(*S*)-hydroxycholesterol, like GW683965A, is able to induce the expression of ABCA1, ABCG1, and apoE in astrocytes and to elevate apoE-mediated cholesterol efflux in astrocytoma but not in neuroblastoma cells. Our observations support the hypothesis that 24(*S*)-hydroxycholesterol participates in an LXR-controlled pathway that regulates cholesterol availability in the brain.

EXPERIMENTAL PROCEDURES

Chemical Reagents—24(*S*)-Hydroxycholesterol was a kind gift from Dr. D. Lutjohann (Bonn University, Germany), and 22(*R*)-hydroxycholesterol was a kind gift from Dr. J. Plat (Maastricht University, The Netherlands). GW683965A was provided by GlaxoSmithKline. The following reagents were purchased from Sigma: 9-*cis*-retinoic acid, leupeptin, aprotinin, phenylmethylsulfonyl fluoride, bovine insulin, human transferrin, putrescine, sodium selenite, and progesterone. ApoA-I and apoE were purchased from Calbiochem. Stocks of 24(*S*)-OH cholesterol (10 mM), 22(*R*)-OH cholesterol (10 mM), and cholesterol (10 mM) were dissolved in ethanol. GW683965A (2 mM) and 9-*cis*-retinoic acid (10 mM) were dissolved in dimethyl sulfoxide.

Cell Culture Experiments—The human astrocytoma cell line CCF-STTG1 and human neuroblastoma cell line SH-SY-5Y were purchased from European Collection of Cell Cultures (Salisbury, UK). CCF-STTG1 and SH-SY-5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum. At 80–90% confluency cells were washed with phosphate-buffered saline (PBS) and treated with different reagents in DMEM/Ham's F-12 medium (1:1) with 10% fetal calf serum for various periods of time as indicated. SH-SY-5Y cells were preincubated for 24 h in the DMEM-Ham's F-12 (1:1) medium without serum containing the N2 supplement (2.5 mg/ml bovine insulin, 10 mg/ml human transferrin (iron-saturated), 0.52 μg/ml sodium selenite, 1.61 mg/ml putrescine, and 0.63 μg/ml progesterone in PBS, pH 7.3). At the end of the treatments conditioned media were collected, and the cells were either lysed in radioimmune precipitation assay buffer (Santa Cruz, CA), or total RNA was isolated as described below for subsequent analyses.

The method of rat primary astrocytes culturing was similar to published procedures (29, 30). Neonatal (postnatal day 1) Lewis rat pups, bred in the animal facilities of Maastricht University, were decapitated, and the neocortex was dissected and cleared of meninges. The tissue was diced in small fragments and incubated in trypsin (0.05% in phosphate-buffered saline) at 37 °C for 15 min. Trypsinization was stopped by adding culture medium, and the tissue was gently centrifuged. The supernatant was discarded, and the pellet was resuspended in 1 ml of culture medium. Single cell dissociation was achieved by 3–5 passes through a 5-ml pipette (Greiner, Germany) and 10–20 passes through a 1-ml pipette (Greiner). Then the tissue was centrifuged very briefly to separate cells from tissue debris. The supernatant containing the cells was then plated into 25-cm^2 cell culture flasks (Corning, NY) at a density of 10^6 cells per flask. Culture medium was refreshed after 4–5 days and every 2 days thereafter. At DIV12 (days *in vitro*) the cultures reached confluence, and contaminating cells were shaken off on a rotary shaker (Rotofix 32, Hettich Zentrifugen). This involves shaking of the flasks for 48 h and refreshment of the medium. At DIV14 the purification is complete and renders >95% glial fibrillary acid protein (GFAP)-immunopositive astrocyte cultures.

Primary murine mixed glial cultures were prepared from postnatal day-1–2 C57Bl/6 mice. Brains from individual animals were placed into ice-cold Hanks'-buffered salt solution (Canadian Invitrogen) containing 6 mg/ml glucose and 10 mM HEPES. Meninges were removed, frontal cortices were dissected, and cells were dissociated by repeated passage through a series of wide to fine bored pipettes. Dissociated cells were plated in DMEM (Invitrogen) with 10% fetal bovine serum, 2 mM L-glutamine (Invitrogen) and 100 units/ml penicillin-streptomycin (Invitrogen) at one 24-well plate per mouse. Cells were cultured in the presence of 5% CO_2 for 12 days when cells were confluent and contained at least 80% astrocytes. Cells were treated with either vehicle-only (ethanol) or increasing concentrations of 24(*S*)-hydroxycholesterol for 24 h. Subsequently, cells were washed once with phosphate-buffered saline, harvested, and lysed using a buffer containing 10% glycerol, 1% Triton X-100, and protease inhibitor (Roche Applied Science) in PBS. Protein concentration was determined by a Dc protein assay (Bio-Rad).

Western Blot Analysis—Cell lysates (25 μg of protein/lane) or conditioned media concentrates (concentrated using Microcon® centrifugal filter device, Millipore, Billerica, MA) were subjected to dodecyl sulfate-10 (SDS) or 12% PAGE and then transferred to Protran nitrocellulose transfer membranes (Schleicher & Schuell) or to polyvinylidene membranes (Millipore). After blocking with 5% nonfat dry milk (Protifar-plus, Nutricia Netherland B.V.) in washing buffer (PBS with 0.5% Triton-X100), the membranes were incubated with antibodies against human apoE (1:500, DAKO A/S, Denmark), murine apoE (Santa Cruz), human glutamine synthetase (1:500, BD Transduction Laboratories), glyceraldehyde-3-phosphate dehydrogenase (Chemicon), or a monoclonal anti-ABCA1 antibody raised against the second nucleotide binding domain (NBD2 of ABCA1) (31) overnight at 4 °C. The membranes were then incubated with peroxidase-conjugated secondary antibodies, after which the results were visualized using ECL reagents (Amersham Biosciences) and autoradiography (LAS 3000, Fuji Photo Film Co., Ltd., Japan). Bands were quantitated by densitometry using NIH Image J.

RNA Isolation and Real-time Quantitative PCR (QRT-PCR) Procedures—Total RNA was isolated using the Trizol method (Invitrogen) according to the manufacturer's instructions. Integrity of RNA was checked by agarose gel electrophoresis, and RNA concentration was measured spectrophotometrically (NanoDrop, Witec AG, Littau, Germany). Real-time quantitative PCR was performed using an Applied Biosystems 7700 sequence detector with 1.6.3 software (PerkinElmer Life Sciences) as previously described (32) with modifications (33). Primer sequences are available upon request. Primers were obtained from Invitrogen. Fluorogenic probes labeled with 6-carboxyfluorescein

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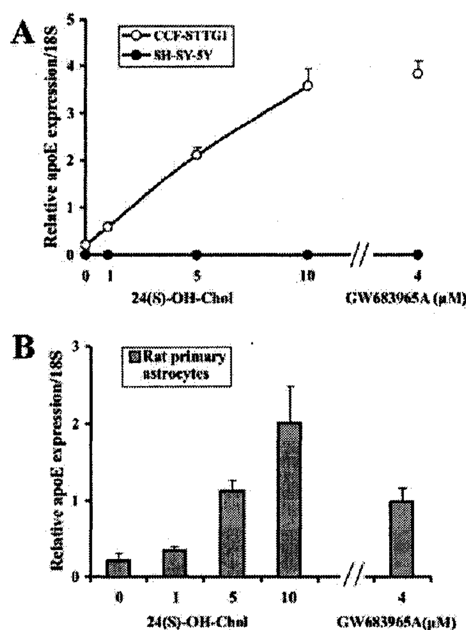

FIGURE 1. **Concentration-dependent effect of 24(S)-hydroxycholesterol (*Chol*) on apoE mRNA levels in CCF-STTG1 and SH-SY-5Y cells and primary rat astrocytes.** Cells were incubated with increasing concentrations of 24(S)-hydroxycholesterol and 4 μM GW683965A compound for a period of 72 h. Total RNA was prepared from cells. Expression levels of apoE in astrocytoma and neuroblastoma cells (*A*) and rat primary astrocytes (*B*) were determined by QRT-PCR and expressed as relative gene expression to 18 S. Values represent the mean ± S.D., $n = 4$ in all groups.

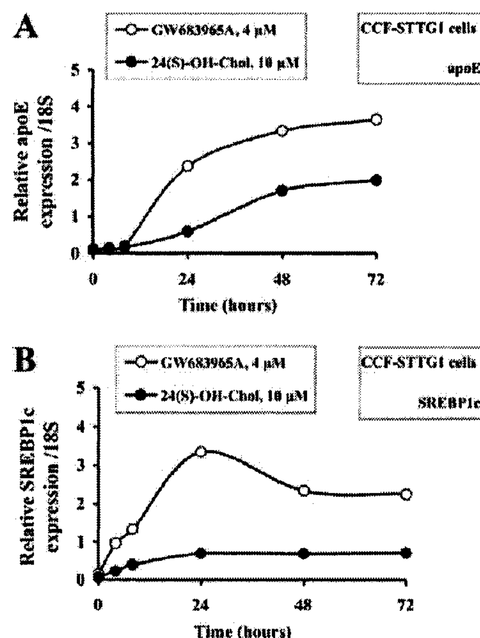

FIGURE 2. **Time-dependent effect of 24(S)-hydroxycholesterol (*Chol*) and GW683965A on apoE and SREBP1c expression in CCF-STTG1 cells.** Cells were incubated in the presence of 10 μM 24(S)-hydroxycholesterol (●) or 4 μM GW683965A (○) for increasing periods of time. Total RNA was prepared from cells. Expression levels of apoE (*A*) and SREBP1c (*B*) were determined by QRT-PCR and expressed as relative gene expression to 18 S. Representative examples of three independent experiments are shown.

(FAM) and 6-carboxytetramethylrhodamine (TAMRA) were made by Eurogentec (Seraing, Belgium).

Efflux Studies—CCF-STTG1 or SH-SY-5Y cells were cultured in DMEM/Ham's F-12 medium (1:1) supplemented with 10% fetal calf serum. After washing with DMEM/Ham's F-12 medium (1:1), cells were loaded with 30 μg/ml [^{3}H]cholesterol (38 Ci/mmol) in DMEM/Ham's F-12 medium (1:1) supplemented with 10% fetal calf serum for 24 h in the presence or absence of 24(S)-hydroxycholesterol or GW683965A. After cells were washed 5 times with PBS-bovine serum albumin 0.2% (w/v), the efflux assay was started by adding 2.5 μg/ml apoE or 5 μg/ml apoA-I in DMEM/Ham's F-12 medium (1:1) to the wells. After 20 h of incubation at 37 °C, the medium was collected and centrifuged. Subsequently, [^{3}H]cholesterol was quantified in the supernatant by liquid scintillation counting. Total cellular [^{3}H]cholesterol was determined after extraction of the cells with 2-propanol. The percentage efflux was calculated by dividing the radioactive counts in the efflux medium by the sum of the counts in the medium and the cell extract.

Statistical Analysis—Values are presented as the mean ± S.D. Statistical significance was determined by comparing means using an unpaired the Student's *t* test, the Mann-Whitney *U* test, and one-way analysis of variance with Newman-Keul's post-test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

The Natural LXR Ligand 24(S)-Hydroxycholesterol Induces ApoE Gene Expression and Protein Levels in Astrocytoma Cells and in Primary Astrocytes but Not in Neuroblastoma Cells—To determine the effect of the natural LXR ligand 24(S)-hydroxycholesterol on apoE expression in astrocytoma and neuroblastoma cells and in primary astrocytes, cells were incubated with increasing amounts of the compound. We found that the incubation with 24(S)-hydroxycholesterol resulted a dose-dependent increase of APOE mRNA levels in astrocytoma cells (CCF-STTG1) but failed to induce APOE expression in neuroblastoma cells (SH-SY-5Y) even after 72 h of incubation (Fig. 1*A*). The synthetic LXR agonist GW683965A (4 μM) also clearly induced APOE expression in astrocytoma cells but not in neuroblastoma cells (Fig. 1*A*), strongly suggesting that 24(S)-hydroxycholesterol exerts its effects via the LXR pathway. Importantly, 24(S)-hydroxycholesterol also induced APOE gene expression in primary rat astrocytes in a dose-dependent manner (Fig. 1*B*), supporting the physiological relevance of this process.

The induction of APOE expression (Fig. 2*A*) by 24(S)-hydroxycholesterol and GW683965A in astrocytoma cells also appeared to be time-dependent, with strong induction already occurring after 24 h and a steady increase up to 72 h of incubation. The established LXR target gene SREBP-1c was also induced by both compounds but displayed a different induction profile compared with apoE (Fig. 2*B*). The expression of APOD, another LXR-target gene that has been suggested to compensate cholesterol transport functions in the absence of APOE (34), was not affected by the natural or synthetic LXR ligand in either cell line (data not shown). Next we determined if induction of APOE mRNA up-regulation resulted in increased protein levels. As shown in Fig. 3*A*, cellular apoE protein levels in astrocytoma cells were also clearly up-regulated by 24(S)-hydroxycholesterol in a concentration-dependent manner. Upon incubation of the cells for increasing periods of time in the presence of 10 μM 24(S)-hydroxycholesterol, apoE protein levels increased up to 48 h and remained fairly constant thereafter up to 72 h (Fig. 3*B*). Similar to 24(S)-hydroxycholesterol, the synthetic LXR agonist GW683965A

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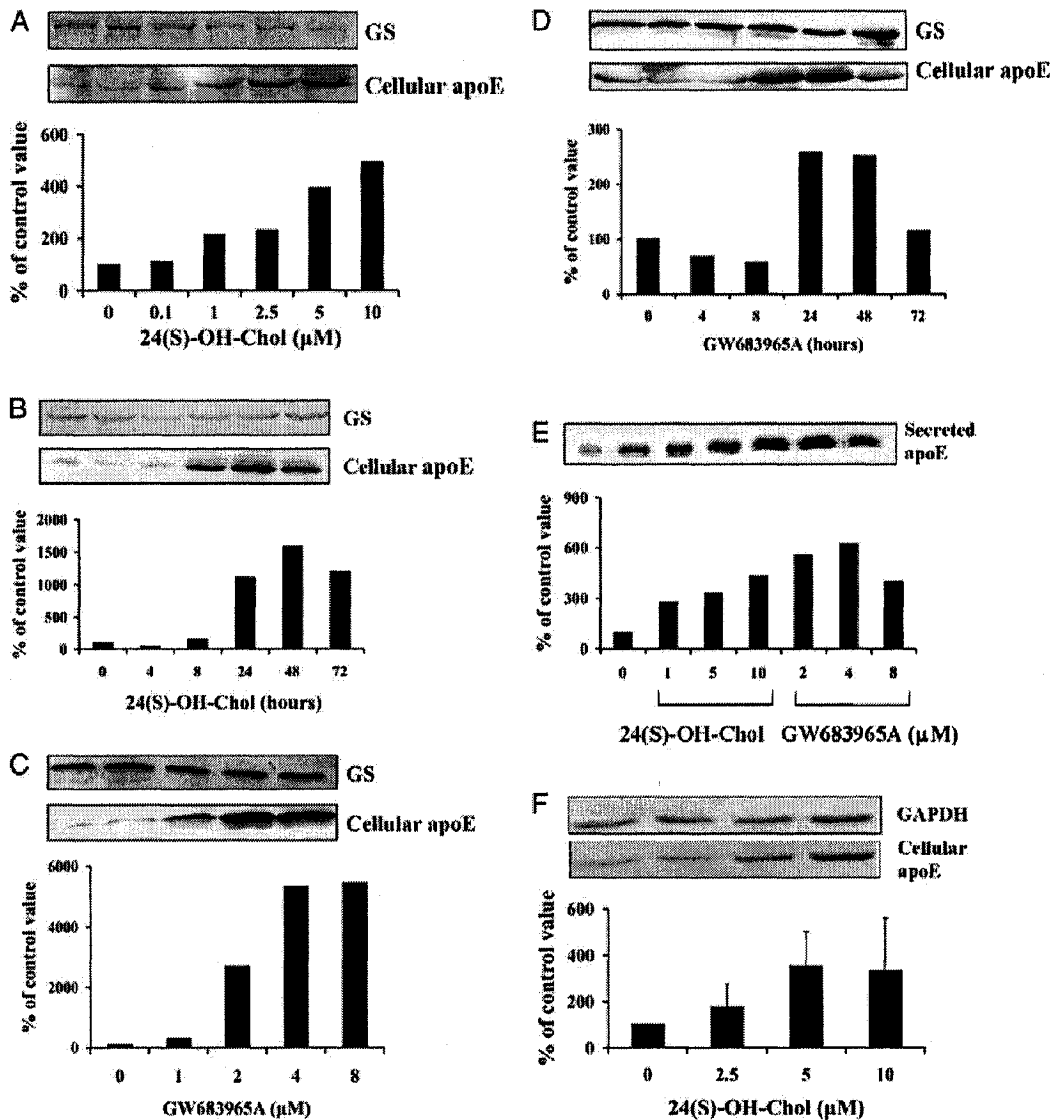

FIGURE 3. **24(S)-Hydroxycholesterol (*Chol*) and GW683965A increase apoE protein levels and apoE secretion in CCF-STTG1 cells.** 24(S)-hydroxycholesterol up-regulates apoE protein levels in murine primary glial cultures. CCF-STTG1 cells were treated either with increasing concentrations of 24(S)-hydroxycholesterol (*A*) and GW683965A (*C*) for a period of 72 h or with 10 μM 24(S)-hydroxycholesterol (*B*) and 4 μM GW683965A (*D*) for increasing periods of time. Conditioned medium was collected after the treatment of CCF-STTG1 cells with different concentrations of 24(S)-hydroxycholesterol and GW683965A for a period of 48 h (*E*). Murine primary glial cultures were treated with increasing concentrations of 24(S)-hydroxycholesterol for 24 h (*F*). Cell lysates and medium concentrates were subjected to Western blot analysis as described under "Experimental Procedures." ApoE expression was measured using a polyclonal antibody against apoE. Bands were quantified by densitometry, normalized to glutamine synthetase (*GS*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Representative experiments of at least three independent experiments are shown.

increased apoE protein levels in astrocytoma cells in a concentration- and time-dependent manner (Fig. 3, *C* and *D*). Secretion of apoE by astrocytoma cells into the medium was also strongly induced by 24(S)-hydroxycholesterol and GW683965A in a concentration-dependent manner (Fig. 3*E*). 24(S)-Hydroxycholesterol also up-regulated cellular apoE protein levels in murine primary mixed glial cultures containing >80% astrocytes (Fig. 3*F*), further substantiating our findings.

Because differences in expression of LXRs may underlie the differential effects of 24(S)-hydroxycholesterol and GW683965A in the two cell types, we next determined their basal expression levels. Significantly higher mRNA levels of LXRα and LXRβ were found in astrocytoma cells compared with neuroblastoma cells (Fig. 4). Also SREBP-1c, an established LXR target gene involved in cholesterol and fatty acid metabolism (35), was expressed predominantly in astrocytoma cells. In contrast, mRNA levels of SREBP-2, another transcription factor involved in control of cholesterol metabolism, were similar in both cells types (Fig. 4*A*). Primary rat astrocytes also displayed a similar transcription profile for these genes (Fig. 4*B*).

24(S)-Hydroxycholesterol Is a Stronger Regulator of Intracellular ApoE Protein Levels in Astrocytoma Cells than Retinoic Acid and Free Cholesterol—24(S)-Hydroxycholesterol was compared with other natural LXR/retinoid X receptor (RXR) ligands with respect to its effect on

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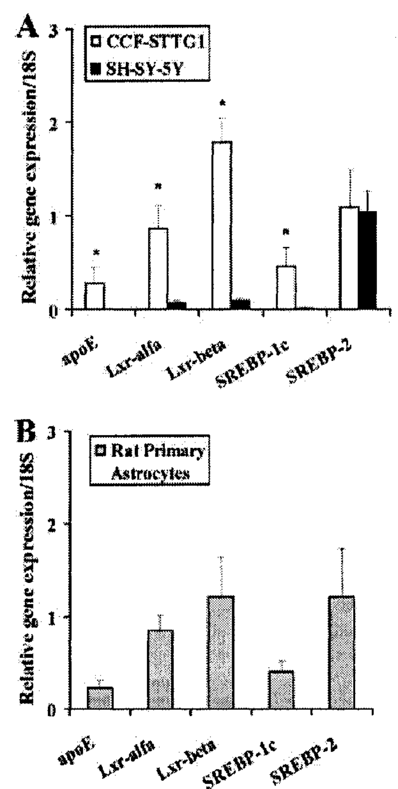

FIGURE 4. **Comparison of the basal expression of apoE, LXRα, LXRβ, SREBP1c, and SREBP-2 in CCF-STTG1 and SH-SY-5Y cells and in rat primary astrocytes.** Cells were incubated for 72 h in DMEM/Ham's F-12 (1:1) medium containing 10% fetal calf serum. Total RNA was prepared from cells. Expression levels of apoE, LXRα, LXRβ, SREBP1c, and SREBP2 in CCF-STTG1 and SH-SY-5Y cells (*A*) and rat primary astrocytes (*B*) were determined by QRT-PCR and expressed as relative gene expression to 18 S. Values represent the mean ± S.D., $n = 4$ in all groups. The Mann-Whitney *U* test was used to determine significant differences in gene expression. The *asterisk* represents $p < 0.01$.

intracellular apoE levels. In accordance with the observation that brain apoE is predominantly synthesized by astrocytes (36, 37), we observed that the basal expression of the APOE gene in astrocytoma cells was substantially higher than in neuroblastoma cells (Fig. 4*A*). In astrocytoma cells, 24(*S*)-hydroxycholesterol and GW683965A are as potent as 22(*R*)-hydroxycholesterol in inducing intracellular and secreted apoE and were considerably more effective than retinoic acid or free cholesterol (Fig. 5, *A* and *C*). Neither 24(*S*)-hydroxycholesterol nor GW683965A or any of the other LXR/retinoid X receptor (RXR) agonists affected apoE synthesis in neuroblastoma cells (Fig. 5*B*), demonstrating that LXR-mediated up-regulation of apoE synthesis and secretion is a pathway specific to astrocytes.

24(S)-Hydroxycholesterol and GW683965A Regulate the Expression of ABC Transporters and Other Genes Related to Cholesterol Metabolism in a Cell Type-specific Manner—ABCA1, ABCG1, and ABCG4 are transporters of cholesterol and/or phospholipids from cells to extracellular acceptors that may also be involved in intercellular lipid transport within the brain (38, 39). Because all three transporters have been identified as LXR target genes present in the brain (40, 41), we examined the effects of 24(*S*)-hydroxycholesterol and GW683965A on their expression. The results presented in Fig. 6*A* show that basal expression of ABCG1 mRNA was comparable in astrocytoma cells and in neuroblastoma cells. The relative expression of ABCA1 mRNA was higher than that of ABCG1 and also 3-fold higher in neuroblastoma than in astrocytoma cells. Likewise, the basal relative expression of ABCG4 mRNA

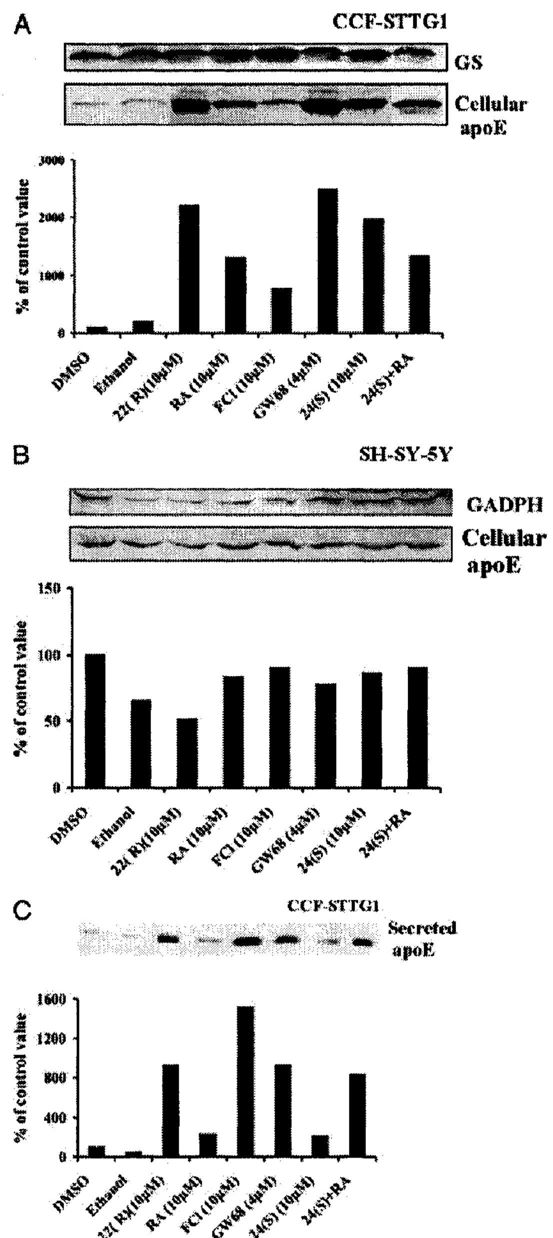

FIGURE 5. **24(*S*)-Hydroxycholesterol is a stronger regulator of apoE synthesis in CCF-STTG1 cells than retinoic acid and cholesterol.** Shown are Western blot analyses of CCF-STTG1 (*A*) and SH-SY-5Y (*B*) cell lysates and conditioned medium from CCF-STTG1 cells (*C*) that were incubated for 72 h in the presence of either 10 µM 24(*S*)-hydroxycholesterol, 22-(*R*)-hydroxycholesterol, 9-*cis*-retinoic acid, free cholesterol (*FCl*), or 4 µM GW683965A. Bands were quantified by densitometry, normalized to glutamine synthetase (*GS*) and glyceraldehyde-3-phosphate dehydrogenase (*GADPH*). Representative experiments are shown; at least three independent experiments were performed for all conditions. *DMSO*, Me_2SO.

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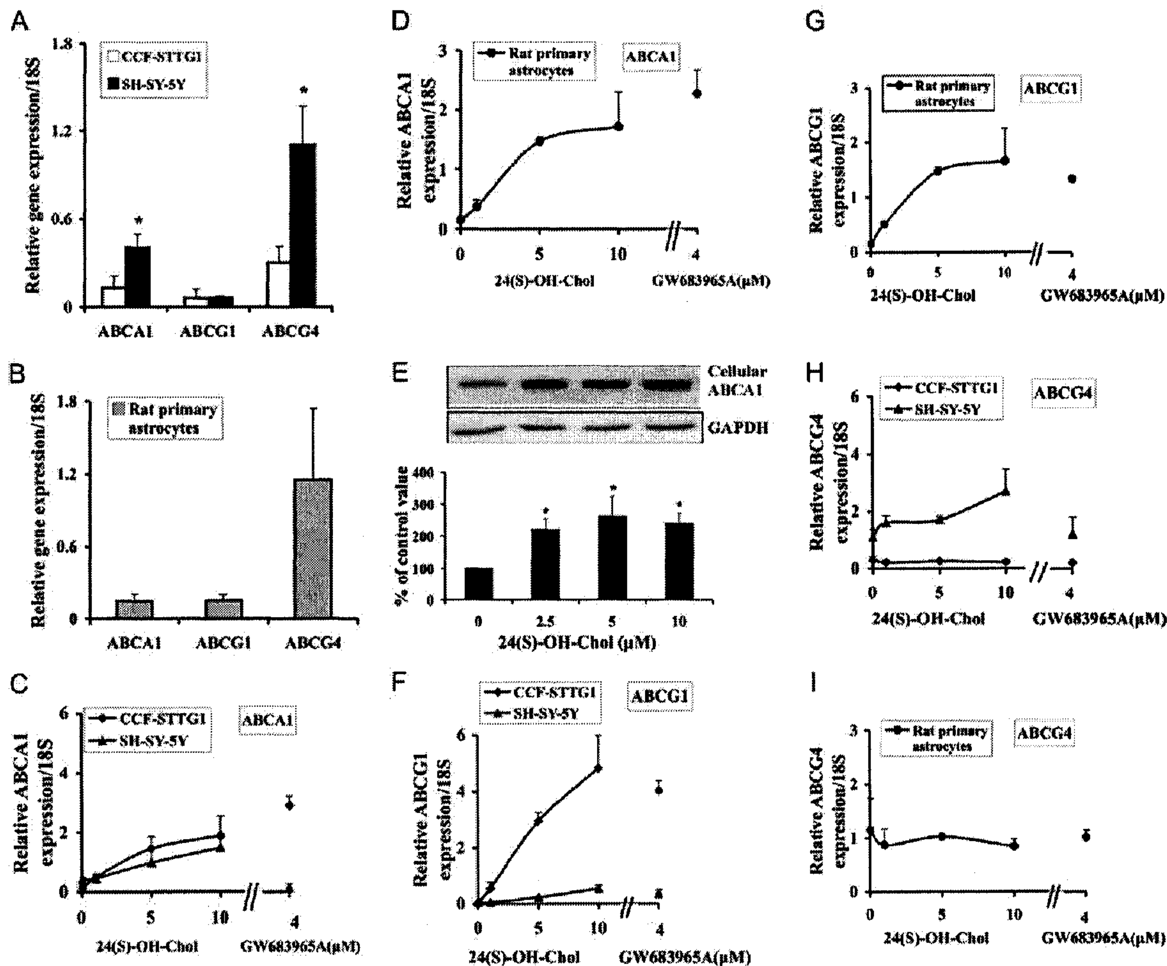

FIGURE 6. **Effect of 24(*S*)-hydroxycholesterol (*Chol*) and GW683965A on mRNA levels of ATP binding cassette transporters in CCF-STTG1 and SH-SY-5Y cells and rat primary astrocytes.** This panel represents a basal gene expression of ATP binding cassette transporters in CCF-STTG1 and SH-SY-5Y cells (*A*) and in rat primary astrocytes (*B*). Total RNA was prepared from the cells as described under "Experimental Procedures." Gene expression relative to 18 S was determined by QRT-PCR. The Mann-Whitney *U* test was used to determine significant differences in gene expression. An *asterisk* indicates that $p < 0.01$. Astrocytoma and neuroblastoma cells (*C*, *F*, and *H*) and rat primary astrocytes (*D*, *G*, *I*) were incubated for 72 h in the presence of 1, 5, or 10 μM 24(*S*)-hydroxycholesterol or in the presence of 4 μM GW683965A. Total RNA was prepared from the cells as described under "Experimental Procedures." Gene expression of ABCA1 (*C* and *D*), ABCG1 (*F* and *G*), and ABCG4 (*H* and *I*) relative to 18 S was determined by QRT-PCR. Protein levels of ABCA1 in whole lysates of primary mixed glial cultures treated with increasing concentrations of 24(*S*)-hydroxycholesterol for a period of 24 h were determined by Western blot and quantified by densitometry. Intracellular glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal protein loading control (*E*). Data represent the mean and S.D. of cellular ABCA1 levels. One-way analysis of variance with Newman-Keul's post-test was used to determine significant differences (the *asterisk* represents $p < 0.01$ compared with vehicle-only).

was significantly higher in SH-SY-5Y cells than in astrocytoma cells (Fig. 6*A*). A pattern of relative ABC transporter expression similar to that in astrocytoma cells was found in primary rat astrocytes (Fig. 6*B*) Incubation with 24(*S*)-hydroxycholesterol resulted in a dose-dependent up-regulation of the expression of ABCA1 mRNA in astrocytoma, in neuroblastoma cells (Fig. 6*C*), and in primary astrocytes (Fig. 6*D*). In contrast, GW683965A induced ABCA1 mRNA only in astrocytoma cells and rat primary astrocytes (Fig. 6, *C* and *D*). Furthermore, 24(*S*)-hydroxycholesterol increased ABCA1 protein levels in murine primary glia (Fig. 6*E*).

ABCG1 mRNA was strongly induced by both 24(*S*)-hydroxycholesterol and GW683965A in astrocytoma cells (Fig. 6*F*), and primary astrocytes also demonstrated a significant induction of ABCG1 mRNA (Fig. 6*G*). In contrast, ABCG1 was unresponsive to either compound in neuroblastoma cells (Fig. 6*F*). Interestingly, ABCG4 was slightly up-regulated by 24(*S*)-hydroxycholesterol but not by GW683965A in neuroblastoma cells, whereas this gene was not affected or even slightly down-regulated in astrocytoma cells and in primary astrocytes (Fig. 6, *H* and *I*). Effects of 24(*S*)-hydroxycholesterol and GW68395A on the expression of the ABC transporters mentioned were maximal at 24 h of incubation in astrocytoma cells (data not shown).

Next we determined the effect of 24(*S*)-hydroxycholesterol or GW683965A on mRNA levels of other genes involved in cholesterol and/or fatty acid metabolism. Incubation of astrocytoma cells with 24(*S*)-hydroxycholesterol resulted in a transient down-regulation of SREBP-2, LDLR, and HMG-CoA reductase mRNA levels. In contrast,

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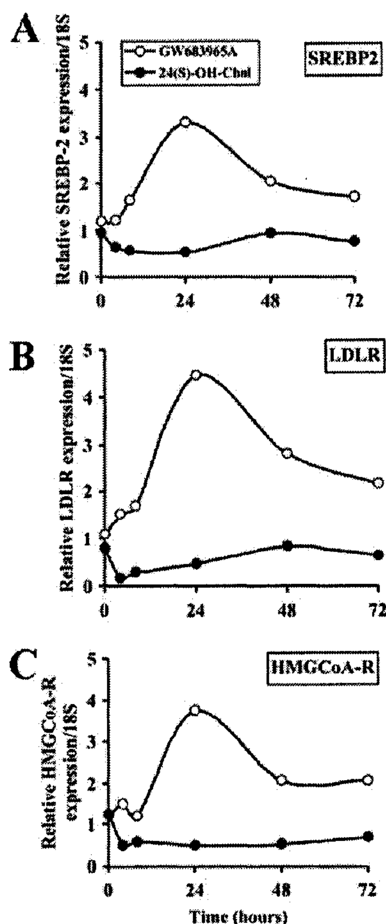

FIGURE 7. **Time-dependent effect of 24(S)-hydroxycholesterol and GW683965A on expression of SREBP2 (A), LDLR (B), HMG-CoA reductase (C) in CCF-STTG1 cells.** CCF-STTG1 cells were incubated in the presence of 10 μM 24(S)-hydroxycholesterol (●) or 4 μM GW683965A (○) for increasing periods of time. Total RNA was prepared from cells, and gene expression relative to 18S was determined by QRT-PCR.

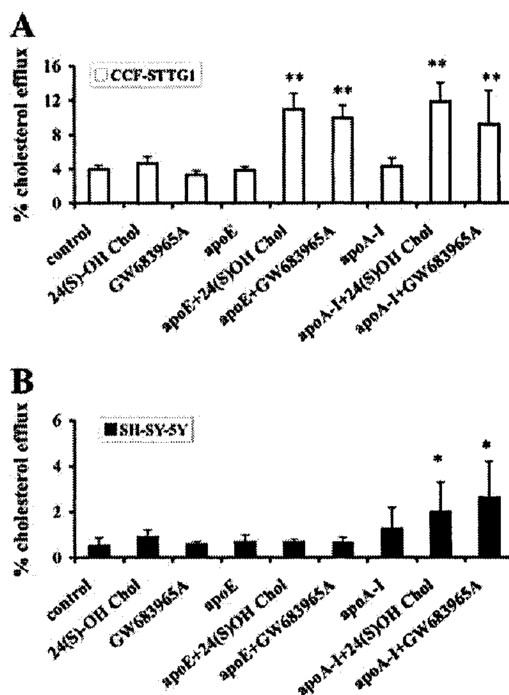

FIGURE 8. **24(S)-Hydroxycholesterol (*Chol*) and GW683965A induce apoE- and apoAI-mediated cholesterol efflux from CCF-STTG1 cells but not from SH-SY-5Y cells.** After loading the cells with [³H]cholesterol for 24 h, the apoE- or apoA-I-mediated cholesterol efflux over a period of 20 h from CCF-STTG1 cells (*A*) and SH-SY-5Y cells (*B*) was determined. [³H]Cholesterol was quantified in medium and in cell extracts. Values are the mean ± S.D. of three independent experiments, each measured in duplicate. Student's *t* test was used to determine significant differences of cholesterol efflux levels. The *single asterisk* represents $p < 0.05$, and the *double asterisk* represents $p < 0.001$.

GW683965A induced a transient increase in the expression of these genes with a maximal effect at 24 h (Fig. 7). SR-BI expression in astrocytoma cells was not affected by either compound (data not shown). Additionally, neither 24(S)-hydroxycholesterol nor GW683965A detectably affected the expression of SREBP-2, HMG-CoA reductase, and LDLR in neuroblastoma cells (data not shown).

24(S)-Hydroxycholesterol Enhances ApoE- and ApoA-I-mediated Cholesterol Efflux from Astrocytoma Cells, whereas Only the ApoA-I-mediated Cholesterol Efflux from Neuroblastoma Is Enhanced—We observed that basal cholesterol efflux from astrocytoma cells was higher than from neuroblastoma cells (~4% compared with 0.5%, respectively; Fig. 8). The addition of 24(S)-hydroxycholesterol induced apoE- and apoA-I-mediated cholesterol efflux from astrocytoma cells to equivalent levels (Fig. 8*A*), and similar effects were observed with synthetic LXR agonist GW683965A. Interestingly, neither compound affected apoE-mediated cholesterol efflux from neuroblastoma cells (Fig. 8*B*) but, rather, specifically induced apoA-I-mediated cholesterol efflux from neuroblastoma cells (Fig. 8).

DISCUSSION

In this paper we addressed the regulation of genes involved in apoE-mediated cholesterol trafficking between astrocytes and neurons, an important process during regeneration and synaptic plasticity (25). We observe that 24(S)-hydroxycholesterol is capable of inducing apoE and ABC transporter expression in astrocytic cells as well as in primary rat and murine astrocytes. In contrast, 24(S)-hydroxycholesterol did not induce expression of these genes in SHSY5Y neuronal cells. Notably, 24(S)-hydroxycholesterol is a natural LXR ligand found in the brain, and elevated levels of 24(S)-hydroxycholesterol levels are often associated with neuronal injury. Our results are consistent with a model in which release of 24(S)-hydroxycholesterol from neurons can induce the secretion of apoE-associated cholesterol from astrocytes. This glial-derived cholesterol would then be available for neuronal uptake during the process of dendritic and axonal extension and regeneration of synapses (26).

Because the potent synthetic LXR agonist GW683965A also induced apoE, ABCA1, and ABCG1 mRNA expression in astrocyte-derived cells, our results suggest that 24(S)-hydroxycholesterol mediates its action through LXR activation. Furthermore, differential expression of the nuclear hormone receptor LXR isoforms, *i.e.* LXRα and -β, between astrocytoma and neuroblastoma cells may also account for the difference in sensitivity to these agonists between astrocytic and neuronal cells. Our results suggest that 24(S)-hydroxycholesterol-mediated induction of ABCG1 and ABCA1 primes the astrocytes to deliver cholesterol to apoE or apoA-I, since their expression was robustly induced

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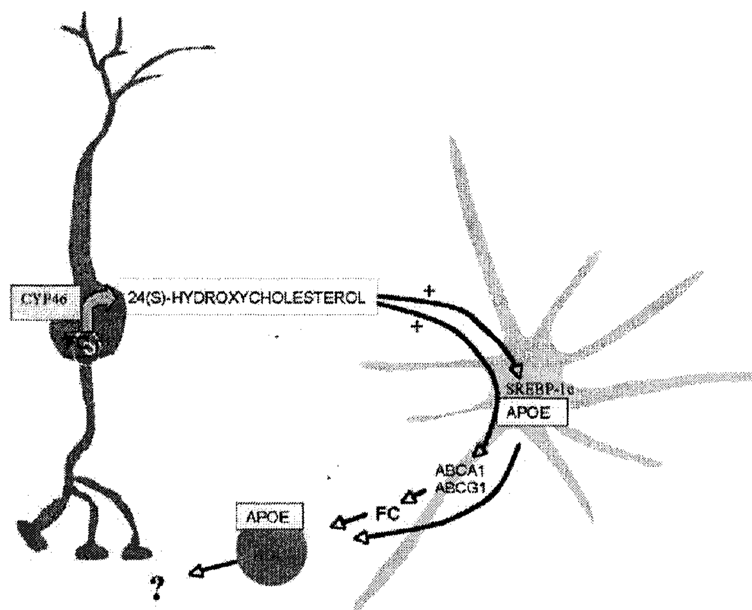

FIGURE 9. **Schematic presentation of the differential effects in astrocytes and neurons of 24(S)-hydroxycholesterol on the expression of apoE and ABC transporters expression as well as apoE-mediated cholesterol efflux.** 24(S)-Hydroxycholesterol up-regulates apoE synthesis as well as apoE-mediated cholesterol efflux in astrocytes but not in neurons. Up-regulation of ABCA1 and ABCG1 in astrocytes by 24(S)-hydroxycholesterol suggests involvement of these transporters in the efflux of cholesterol. *HDL*, high density lipoprotein.

by both compounds. In neuroblastoma cells, neither of the two compounds stimulated apoE-mediated cholesterol efflux, but both compounds induced apoA-I-mediated cholesterol efflux, probably involving ABCA1 actions.

It has been proposed that, after differentiation of astrocytes, neurons reduce their endogenous cholesterol synthesis and rely predominantly on cholesterol delivery by astrocytes via lipoprotein-like particles that contain astroglia-derived apoE (47). Cholesterol delivery may at least in part participate in regulating the number of synapses formed (42). A continuous turnover of cholesterol in neurons facilitates cell ability for efficient and quick adaptation of cholesterol homeostasis required for dynamic structural changes of neurons, their extensions, and their synapses during synaptic plasticity (43). Conversion of cholesterol into 24(S)-hydroxycholesterol, which is also a brain-specific LXR ligand, by CYP46 represents a major route for cholesterol turnover in neurons (44).

A common feature that astrocytes share with macrophages and adipocytes is their large content of free cholesterol (45, 46). In agreement with what has been reported for macrophages (47), we found that activation of LXR by 24(S)-hydroxycholesterol or GW683965A stimulates cellular cholesterol efflux through the coordinated regulation of ABCA1, ABCG1, and apoE. Some aspects of these pathways may be specific to astrocytes, however, because apoD was unresponsive to LXR agonists in astrocytes, whereas it is induced in adipocytes (48).

GW683965A up-regulated the expression of HMG-CoA reductase, LDLR, and SREBP2 in astrocytoma cells, supposedly to maintain cellular cholesterol homeostasis during excessive loss by efflux. However, 24(S)-hydroxycholesterol down-regulated the expression of these genes. It is long since known that oxysterol s reduce the activity of HMG-CoA reductase and are more potent inhibitors of cholesterol synthesis than cholesterol (49). However, the mechanisms by which cholesterol and oxysterols reduce cholesterol synthesis differ (50). Cholesterol directly interferes with SREBP cleavage-activating protein, inducing a conformational change that prevents the processing of SREBP to its active form. Oxysterols have similar effects without directly interacting with SREBP cleavage-activating protein. Thus, 24(S)-hydroxycholesterol may not exert its effect exclusively via the LXR pathway. The observation that 24(S)-hydroxycholesterol, but not GW683965A, enhanced the expression of ABCG4 in neurons suggests that LXR-independent pathways may be involved. Apparently, cholesterol efflux from astrocytes is not directly driven solely by the rate of cholesterol biosynthesis, because efflux was induced to a similar level by 24(S)-hydroxycholesterol and GW683965A. A concomitantly enhanced cholesterol efflux, up-regulation of ABCA1 and ABCG1, and impaired synthesis of cholesterol has recently also been reported as a consequence of statin treatment in macrophages (47).

Numerous studies have demonstrated that ABCA1 is necessary for the efflux of cellular cholesterol to lipid-poor apoA-I (51). Recently, ABCA1 was found to facilitate the efflux of central nervous system cholesterol to apoE as the absence of ABCA1 compromised apoE secretion from both astrocytes and microglia. In addition, apoE that is present in the cerebrospinal fluid of ABCA1-deficient animals is poorly lipidated (38, 52). In contrast to ABCA1, ABCG1 and ABCG4 are thought to facilitate the efflux of cholesterol to high density lipoprotein rather than to lipid-poor apolipoproteins (53, 54). A relationship between ABCG1 and the secretion of apoE was suggested by the observation that treatment of macrophages with antisense oligonucleotides to ABCG1 decreased the efflux of cholesterol and phospholipids to high density lipoprotein and, surprisingly, also the secretion of apoE (53, 55). Although ABCG1 and ABCG4 may function both as homodimers and heterodimers (56, 57), expression of ABCG1 and ABCG4 overlaps in some but not all tissues assayed (54), which may indicate different functions in different tissues. The expression of ABCG4 appears to be largely restricted to nervous tissue (56). Our results strongly suggest that apoA-I-mediated cholesterol efflux from astrocytes involves ABCA1 and that apoE mediates cholesterol efflux via ABCG1 and possibly also ABCA1, but not via ABCG4. Although 24(S)-hydroxycholesterol also stimulated apoE synthesis and secretion from astrocytic cells, it remains to be established why 24(S)-hydroxycholesterol treated resulted in observable cholesterol efflux only in the presence of exogenous apoE or

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apoA-1. A possible explanation may be that the levels of endogenous apoE secreted from 24(*S*)-hydroxycholesterol-treated cells is >100-fold lower than the concentrations of exogenous apoE added as a lipid acceptor under our experimental conditions. Alternatively, endogenously secreted apoE may already be lipidated and thereby act a less efficient cholesterol acceptor.

Neurons are thought to dispose their cholesterol by conversion into 24(*S*)-hydroxycholesterol, which is more polar than cholesterol and, as a result, may easily traverse the blood-brain barrier and perhaps the neuronal plasma membrane itself (4). However, how oxysterols are transported across membranes and through the intracellular water phase is not yet known. The selective up-regulation of ABCG4 in neuroblastoma cells by 24(*S*)-hydroxycholesterol suggests a possible role for this transporter in oxysterol transport. Both 24(*S*)-hydroxycholesterol and also GW683865A enhanced apoA-I-mediated cholesterol efflux from neuronal cells, suggesting this is another neuronal pathway to dispose of cholesterol. However, GW683965A had only a limited effect on ABCA1 expression in these cells. Rebeck *et al.* (57) recently reported up-regulation of neuronal ABCA1 expression by the synthetic LXR ligand T0901317. A role for apoA-I in the disposal of cholesterol from neurons is in line with its well known role in so called "reverse cholesterol transport." ApoA-I is present in brain and in cerebrospinal fluid and has been detected in senile plaques in AD patients (58, 59). So far apoA-I synthesis within the brain has only been ascribed to endothelial cells of the blood brain barrier (60). It remains to be established why 24(*S*)-hydroxycholesterol does not alone, but only in concert with apoE or apoAI increase cholesterol efflux from astrocytes. A possible explanation may be the relatively small amounts (<100-fold) of apoE that are secreted by the cells in comparison with the amount of apoE that is added as cholesterol acceptor. Alternatively, secreted apoE may be lapidated and thereby become a less efficient cholesterol acceptor.

LXR isoforms differ in their pattern of expression (61). In the brain LXRβ levels are 2–5-fold higher than in the liver, whereas LXRα levels are 3.5–14-fold lower than in the liver (62–64). However, 24(*S*)-hydroxycholesterol and GW683965A up-regulated LXRα but not LXRβ expression in astrocytoma cells (data not shown), similar to what has been reported for macrophages and adipocytes, but not liver and muscle (65, 66). These results suggest the possibility that the autoregulation of LXRα that has been suggested to occur in adipocytes to coordinate expression of target genes such as APOE (66) may also occur in brain.

In conclusion, our results provide evidence indicating that 24(*S*)-hydroxycholesterol acts as a signaling molecule that induces the apoE-mediated cholesterol efflux from astrocytes but not from neurons (Fig. 9). Our findings also suggest a role for ABCA1 and ABCG1 in mediating cholesterol efflux from astrocytes. Thus, in the intact brain, 24(*S*)-hydroxycholesterol derived from neurons may signal astrocytes to increase production of lipidated apoE particles in order to supply neurons with additional cholesterol during synaptogenesis or neuritic remodeling. Moreover alterations in the transcriptional regulation role of 24(*S*)-hydroxycholesterol on apoE-mediated cholesterol efflux may affect the progression of neurodegenerative diseases including AD.

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Appendix 1.2: The cholesterol transporter ABCG1 modulates the subcellular distribution and proteolytic processing of β-amyloid precursor protein

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THE CHOLESTEROL TRANSPORTER ABCG1 MODULATES THE SUBCELLULAR DISTRIBUTION AND PROTEOLYTIC PROCESSING OF β-AMYLOID PRECURSOR PROTEIN

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Running Title: ABCG1 regulates APP processing

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Abbreviations:
Amyloid precursor protein (APP), high-density lipoprotein (HDL), Alzheimer's Disease (AD), Down Syndrome (DS), amyloid-β (Aβ), C-terminal fragment (CTF)

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ABSTRACT

Although intracellular cholesterol levels are known to influence the proteolysis of beta-amyloid precursor protein (APP), the effect of specific genes that regulate cholesterol metabolism on APP processing remains poorly understood. The cholesterol transporter ABCG1 facilitates cholesterol efflux to high-density lipoprotein (HDL) and is expressed in brain. Notably, the human ABCG1 gene maps to chromosome 21q22.3, and individuals with Down Syndrome (DS) typically manifest with Alzheimer's Disease (AD) neuropathology in their 30s. Here we demonstrate that expression of ABCG1 enhances amyloid β protein (Aβ) production in transfected HEK cells in a manner that requires functional cholesterol transporter activity. ABCG1-expressing cells also exhibit elevated sAPPα and sAPPβ secretion and display increased cell surface-associated APP. These results suggest that ABCG1 increases the availability of APP as a secretase substrate for both the amyloidogenic and nonamyloidogenic pathways. *In vivo,* ABCG1 mRNA levels are 2-fold more abundant in DS brain compared to age- and sex- matched normal controls. Finally, both Aβ and sAPPα levels are elevated in DS cortex relative to normal controls. These findings suggest that altered cholesterol metabolism and APP trafficking mediated by ABCG1 may contribute to the accelerated onset of AD neuropathology in DS.

KEYWORDS

ABCG1, Alzheimer's Disease, Amyloid precursor protein, Aβ, Down Syndrome

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INTRODUCTION

Alzheimer's Disease (AD) is the most common form of senile dementia and affects approximately 50% of persons over 85 years of age [1]. Age is the biggest risk factor for AD and over 95% of cases have no known cause. However, approximately 5% of AD cases result from mutations within genes that produce a peptide known as amyloid β-peptide (Aβ), which accumulates as amyloid deposits in the parenchyma and cerebral blood vessels in the AD brain and serves as one the major neuropathological hallmarks of AD [1]. Amyloid plaques are composed mainly of fibrillar aggregates of Aβ peptides that are derived from β-amyloid precursor protein (APP) by proteolytic cleavage. Most APP molecules are cleaved by α-secretase at a site within the Aβ domain to release the neurotrophic ectodomain of APP, a process that precludes the generation of Aβ. In contrast, cleavage of APP by β- and γ-secretases generates the Aβ peptides that found in amyloid plaques [2]. Factors that enhance Aβ production or delayed Aβ clearance may therefore contribute to the pathogenesis of AD.

Cholesterol is increasingly recognized to play a key role in AD [3]. Many groups have reported that high intracellular cholesterol levels result in enhanced release of Aβ *in vitro* and *in vivo* [4-7] whereas low intracellular cholesterol levels favor processing of APP through the nonamyloidogenic α-secretase pathway and decrease Aβ production [8-13]. Several of these studies have relied upon pharmacological or chemical manipulation of absolute intracellular cholesterol levels to investigate the relationship between cholesterol and APP processing. However, the relationship between cholesterol and Aβ metabolism is considerably more complex than simply correlating the absolute level of total intracellular cholesterol with APP metabolism. For example, APP and all of the components of secretase enzymes are integral membrane-bound proteins, and aberrant cholesterol transport is known to affect the subcellular distribution of presenilins [7,14]. Furthermore, β-secretase cleavage of APP is dependent on the association of APP with BACE1 in lipid rafts [13], and the proteolytic activity of γ-secretase takes place within the hydrophobic lipid membrane environment [15], suggesting that the subcellular distribution of cholesterol may influence the association of APP with secretases as well as modulate secretase activity. Intriguingly, Aβ-positive synaptic terminals isolated from AD cortex have recently been reported to have 33% more cholesterol than Aβ-negative terminals [16], supporting the view that cholesterol distribution plays an important role in human AD. The transmembrane distribution of cholesterol may also play a key role in APP processing, as wild-type mice treated with simvastatin, lovastatin, and atorvastatin all exhibit reduced endogenous Aβ levels associated with a shift of cholesterol from the cytofacial leaflet to the exofacial leaflet of the plasma membrane rather than with its total cholesterol content [17]. The exofacial leaflet of synaptic membranes in human apolipoprotein E4 (apoE4) knock-in mice has also been reported to contain a 2-fold increase in cholesterol content

compared to apoE3 knock-in animals [18]. These observations suggest that factors that regulate sterol content and trafficking within cells may play key roles in APP processing and contribute to the pathogenesis of AD.

The physiological regulation of intracellular cholesterol levels is mediated by a network of genes involved in sterol homeostasis, and the specific effect of many of these genes on APP processing is not well understood. For example, members of the ABCA and ABCG classes of ATP-binding cassette transporters are believed to act as critical gatekeepers of cholesterol homeostasis [19-21]. ABCA1 is essential for the efflux of excess intracellular cholesterol onto lipid-free or lipid-poor apolipoproteins [22], and we and others have recently shown that ABCA1 is a key regulator of apoE levels and lipidation in the brain and influences amyloid burden but not Aβ production in vivo [23-28].

ABCG1 is the founding member of the ABCG subclass of ABC transporters [21,29], and is widely expressed in several peripheral tissues as well as in grey and white matter in postnatal murine brain [30-32]. Biochemically, ABCG1 facilitates cholesterol efflux to high density lipoprotein (HDL) but not to lipid-free apoA-I [33,32], and redistributes intracellular cholesterol to plasma membrane domains that are accessible by cholesterol oxidase [34]. Both the cholesterol efflux and distribution activities are present when ABCG1 is selectively expressed in cells, demonstrating that ABCG1 can function as a homodimer [32-34]. Deficiency of ABCG1 in mice results in accumulation of sterols within liver and macrophage-rich tissues when animals are challenged with a high-fat, high-cholesterol diet [35]. ABCG1 is also highly expressed in brain and has been proposed to be a better correlate of cholesterol efflux from glia than ABCA1 [30-32,36].

Intriguingly, the human *ABCG1* gene resides on chromosome 21 [37-40], suggesting that it may be of interest for some of the clinical phenotypes associated with Down Syndrome (DS). DS is caused by inheritance of an extra copy of all or part of chromosome 21 and occurs in approximately 1 in 700 live births. Most individuals (95%) with DS are trisomic for the entire chromosome 21, which contains 337 genes whose individual roles in the syndrome are largely unknown [41]. Analysis of DS individuals with partial trisomy 21 has shown that a "critical region" between loci D21S58 and D21S42 accounts for mental retardation and most of the facial features of DS [42-45].

One prominent phenotype in DS is the inevitable development of AD neuropathology including parenchymal and cerebrovascular amyloid plaques and neurofibrillary tangles by the mid-late 30s [46,47]. This is decades earlier than the general population, who typically exhibit signs of AD in the mid-late 70s [48,47]. Onset of clinical dementia in DS is age-dependent, with prevalence rates of approximately 9% between 40-49 years, 36% between 50-59 years, and 55% between 60-69 years [49].

The human *APP* gene maps to chromosome 21q21.3, and two seminal observations demonstrate the pivotal role of *APP* gene dose in determining the age of onset of AD. First, excess APP is required

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for the accelerated onset of AD in DS [50]. This was shown by the identification of a 78-year old DS subject who exhibited no amyloid deposition upon autopsy and who had partial trisomy 21 in which the chromosomal breakpoint excluded *APP* [50]. More recently, five independent kindreds have been identified that contain a duplication of the *APP* locus in the absence of mental retardation and other aspects of DS [51]. These families all exhibit autosomal dominant early-onset AD with an average clinical age of onset of 52 years, and who exhibit abundant amyloid deposition in the parenchyma and cerebrovasculature [51]. Taken together, these studies conclusively demonstrate that *APP* gene dose critically regulates the age of onset of AD.

However, these studies do not rule out the possibility that other genes on chromosome 21 may also contribute to the decreased age of onset of AD neuropathology in DS subjects. Notably, there is evidence for poorly understood locus on chromosome 21 that affects the risk and age of onset for sporadic AD [52], and it is possible that inheritance of extra copies of genes near this region may functionally synergize with excess APP and provide a better explanation for the greatly accelerated onset of AD neuropathology in DS than APP gene dose alone.

We therefore hypothesized that genes on chromosome 21 with known roles in lipid metabolism may be good candidates to participate in the development of early onset AD neuropathology in DS. The human *ABCG1* gene maps to chromosome 21q.22.3 within the DS critical region and near the linkage peak that may affect onset and risk in sporadic AD [44,52]. Here we show that the functional activity of ABCG1 as a cholesterol transporter influences the subcellular distribution and proteolytic processing of APP. Furthermore, we provide evidence of elevated ABCG1 expression and increased proteolytic products of APP in DS cortex compared to normal controls. Our findings suggest the possibility that ABCG1 may contribute to the accelerated onset of AD neuropathology in DS.

MATERIALS AND METHODS

Cell culture and transfection. Human embryonic kidney 293 (HEK293) cells stably expressing human APP_{695} containing the Swedish mutation (HEK-APPswe cells) were cultured in complete growth medium (Dulbecco's Modified Eagle Media (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml of penicillin-streptomycin, and 200 µg/ml geneticin (all reagents Canadian Life Technologies). Cells were transfected with a human ABCG1 cDNA (Image Consortium), a murine ABCG1 cDNA, or empty vector using Fugene (Roche) according to the manufacturer's recommendations. Each of the transient transfection assays presented here were performed using at least three independent transfections, each with Western blotting controls for ABCG1 to confirm reproducible and equivalent transfection efficiency across experiments.

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Measurement of Aβ and sAPP species. Secreted human Aβ1-40 and Aβ1-42 was measured by ELISA (Biosource). Experiments involving transfected HEK-APPswe cells were performed in complete growth media containing serum except for those correlating ABCG1 cholesterol efflux activity with Aβ secretion (Fig 2). Media was changed 24h after transfection and conditioned for 6-32 h. Conditioned media was collected, mixed with Complete Protease Inhibitor (Roche) and frozen at -80°C until required. Samples were thawed only once. ELISA results, expressed as pg/ml, were normalized to total cellular protein to correct for variations in cell number. Time course experiments were conducted such that less than 10% of the total medium was removed over the complete experiment in complete growth media. The rate of Aβ secretion was determined using Vernier Logger Pro (version 3.3) to generate quadratic curves to fit the data according to the criteria for the least possible slope error. The derivatives of these functions were evaluated over the interval [0,10h]. Aβ levels measured during cholesterol efflux assays were obtained from cells conditioned for 6 h in DMEM, 0.2% delipidated BSA to maintain the identifical conditions used in the cholesterol efflux assay (see below). For measurement of sAPP species, culture supernatants were normalized for total cellular protein to correct for variations in cell number and immunoblotted with 6E10 (Chemicon) to detect sAPPα and 10321 (Phoenix Biotech) to detect sAPPβ.

Purification of HDL. HDL was purified by KBr density gradient ultracentrifugation from plasma obtained from normolipidemic human donors. Fractions corresponding to HDL_2 (1.063-1.125 g/ml) and HDL_3 (1.125-1.225 g/ml) were collected, pooled and dialysed against 15 mM NaCl, 0.1 mM EDTA overnight, followed by filter sterilization. Protein levels were determined by Lowry assay.

Cholesterol efflux assay. Cells were seeded at 250,000 cells/well in 24 well-plates and labelled with 1 μCi/ml of ^{3}H-cholesterol (New England Nuclear) for 18-24 h during transfection in complete growth medium. Labelled and transfected cells were then washed once with serum-free DMEM, and 25 μg/ml of $HDL_{2/3}$ was added as a lipid acceptor in serum-free DMEM containing 0.2% delipidated BSA. Media was collected 6h later and centrifuged at 8000 rpm to remove cell debris. Cells were lysed with 50 μl of 0.1 M NaOH and 0.2% SDS and incubated at room temperature for 20 min. 50 μl of media and cell lysate were added to scintillation plates and counted. The percent cholesterol efflux was calculated as the total counts in the medium divided by the sum of the counts in the medium plus the cell lysate [53]. Parallel wells treated under the identical conditions were used for analyses of secreted Aβ levels.

Western blotting. HEK-APPswe cells were lysed in 10% glycerol, 1% TritonX-100 and Complete protease inhibitor (Roche) in phosphate buffered saline (PBS), and centrifuged for 5 min at 9000 rpm.

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Equal amounts of protein, determined by Lowry assay, were resolved through 7.5% or 10% SDS polyacrylamide gels, transferred to polyvinylidene fluoride membranes (Millipore), and immunodetected with 6E10. Nitrocellulose membranes (Millipore) were used in conjunction with anti-ABCG1 (Novus). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Chemicon) was used as an internal protein loading control. Blots were developed using enhanced chemiluminescence (Amersham) according to the manufacturer's recommendations.

For analyses of APP C-terminal fragments (CTFs), cells were lysed in RIPA buffer consisting of 20mM Tris-HCl pH 7.4, 5mM EDTA, 50mM NaCl, 10mM Na pyrophosphate, 50mM NaF, 1% NP40 and Complete Protease Inhibitor. Lysates were sonicated for 20 sec, centrifuged for 5 min at 11,000 x g, and the supernatants were collected. Thirty micrograms of protein were loaded per lane on 4%-10%-17% step gradient Tris-Tricine gels with an anode buffer consisting of 0.2M Tris-HCl pH 8.9, and a cathode buffer consisting of 0.1M Tris HCl pH 8.4, 0.1M Tricine and 0.1% SDS. Following electrophoresis, proteins were transferred to nitrocellulose membranes and probed with anti-APP C-terminal (Sigma) antibodies to detect CTFα or CTFβ.

For analysis of APP and ABCG1 protein expression in tissues, total membranes were purified as described [54]. Tissues were homogenized in 5 volumes of lysis buffer (50 mM mannitol, 2 mM EDTA, 50 mM Tris HCl pH 7.6, and Complete protease inhibitor), and centrifuged at 500 x g to pellet nuclei and debris. Between 400-450 μl of supernatant was layered onto 600 μl of fractionation buffer (300 mM mannitol, 2 mM EDTA, 50 mM Tris HCl pH 7.6) and centrifuged at 100,000 x g for 45 min to pellet total membranes. Membranes were resuspended in 150-200 μl of lysis buffer. SDS was added to a final concentration of 1% prior to SDS-PAGE and immunoblotting with antibodies against APP, ABCG1, and NaK-ATPase as an internal loading control (Novus).

Biotinlyation assay. HEK-APPswe cells were transfected with vector, murine ABCG1, or human ABCG1 for 24h. Cells were washed twice with cold PBS, and treated with 1mg/ml sulfo-NHS-Biotin (Pierce) in PBS for 30 min at 4°C, washed twice with cold PBS, and quenched with cold 3.75 mg glycine/ml PBS. Cells were solubilized in with lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Triton-X 100, and Complete Protease Inhibitor) for 30 min on ice, sonicated, and centrifuged at 14,000 rpm for 5 min at 4°C. Ten percent of the lysate was saved for analysis of total APP. Streptavidin-agarose beads (Pierce) were washed twice in lysis buffer, added to the remaining lysate, and rocked at 4°C overnight. Beads were collected by centrifugation and washed three times in 10 volumes of lysis buffer. Total and cell surface fractions were separated by SDS-PAGE and immunodetected for APP and actin as a loading control.

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Density gradient fractionation. HEK-APPswe cells were transfected with vector or ABCG1 for 24h. Cells were harvested, lysed, and fractionated over a continuous 0.58-to-1.1mol/L sucrose density gradient as described [55,56].

Human tissues. Frozen human post-mortem control, DS, and AD frontal cortex tissue samples were obtained from the University of Maryland Brain and Tissue Bank for Developmental Disorders (Baltimore, Maryland, NIH contract N01-HD-1-3138), and the University of British Columbia Kinsman Laboratory Brain Bank (generously provided by Dr. Pat McGeer) in accordance with University of British Columbia and BC Children's Hospital clinical ethical approval. Each DS and AD sample was individually matched to a control for age, sex, and wherever possible, ethnic background and post-mortem interval (Table 1).

RNA isolation and analysis. RNA from human brain tissues was extracted using Trizol (Invitrogen) according to the manufacturer's protocol. RNA samples were treated with DNAseI prior to cDNA synthesis. cDNA was generated 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 ABCG1. Sequences are: Human ABCG1 forward (5' ACACCATCCCCACGTACCTA 3') and reverse (5' GATGACCCCTTCGAACCCA 3'), human APP forward (5'GCTGGCTGAACCCCAGATT 3') and reverse(5' CCCACTTCCCATTCTGGACAT 3') (56), and human GAPDH forward (5' CCTGCACCACCAACTGCTTA 3') and reverse (5' CATGAGTCCTTCCACGATACCA 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 GAPDH and analysed with 7000 system SDS software v1.2 (Applied Biosystems) using the relative standard curve method.

ELISA procedures to measure sAPPβ, sAPPα, full length APP, and Aβ in brain homogenates: Human brain homogenates prepared in 5.5M guanidine HCl were diluted 25-fold into PBS, containing 2% BSA and 0.05% Tween-20. Purified human recombinant sAPPβ, sAPPα, and full length APP695 were used for standard curves in each ELISA for quantitation. Fifty microliters of each sample were loaded onto a half-area ELISA plate, pre-coated with 8E5, and incubated at 4°C overnight. The sAPPβ fragments were captured with the rabbit polyclonal 192wt (against peptide epitope ISEVKM). The sAPPα fragments were captured with the rabbit polyclonal 3436 (against peptide epitope YEVHHQK). Full length APP was captured with the rabbit polyclonal Zymed anti-APPβ antibody. Goat anti-rabbit

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IgG conjugated with HRP (Horse Radish Peroxidase) was used as reporting antibody. Human Aβ was quantified in guanidine solubilized extracts from brains as previously described [57].

ABCG1-deficient mice: ABCG1-deficient mice were obtained from Deltagen, CA. 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 (IRES)-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. Chimeric animals were generated using embryonic stem cells derived from the 129/OlaHsd genetic background, and were backcrossed to C56Bl/6 mice for at least 7 generations before use. 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.

Histology: Wild-type and ABCG1-hemizygous mice were perfused with PBS. Brains were postfixed in 2% paraformaldeyde in PBC for 24h, rinsed with PBS, and cryoprotected in 20% sucrose in PBS overnight at 4°C. Frozen coronal sections (40 μm) were prepared on a cryostat and mounted on Superfrost Plus (Fisher) slides. Slides were immersed in 1 μg/ml X-gal (5-bromo-4-chloro-3-indoyl β-D-galactosidase) for 2h at 37°C and counterstained with Neutral Red. Brightfield images were captured on a Zeiss Axioplan microscope.

Statistical analysis. Data were analysed by Student's t test or one-way ANOVA with a Newman-Keuls posttest using GraphPad Prism (version 4.0) software. All *in vivo* data were conducted with the rater blinded to genotype.

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RESULTS

ABCG1 increases Aβ levels. To determine whether ABCG1 affects the secretion of Aβ peptides, HEK293 cells stably expressing APP695 containing the Swedish mutation (HEK-APPswe cells) were transiently transfected with empty vector or human ABCG1 cDNA, and the levels of Aβ40 and Aβ42 released into the media were measured over time. We observed a consistent increase in secreted Aβ40 and Aβ42 levels throughout the course of these experiments. For example, the levels of Aβ40 secreted from HEK-APPswe cells expressing ABCG1 was 1.8 fold greater than the vector-only control at t=10h ($p<0.01$, N=2) (Fig 1A), and that of Aβ42 was 2.1 fold greater than the vector control at t=10h ($p<0.05$, N=2) (Fig 1B). Western blot analysis demonstrated that ABCG1 was undetectable in vector-transfected HEK-APPswe cells, but was abundant in cells expressing ABCG1 (Fig. 1C). These data show that the levels of Aβ released from HEK-APPswe cells is augmented in the presence of ABCG1.

Functional ABCG1 is required for increased Aβ secretion. To determine whether the enzymatic function of ABCG1 as a cholesterol transporter [32-34] is required for elevated Aβ secretion, HEK-APPswe cells were transfected with empty vector, wild-type ABCG1, or ABCG1 containing a S220G mutation in the ATP-binding cassette signature motif that is conserved in mouse, rat, dog, and *Drosophila melanogaster*. Cholesterol efflux assays showed that both functional ABCG1 and the presence of HDL were required to observe an increase in cholesterol efflux beyond baseline levels observed in vector-transfected HEK-APPswe cells (Fig 2A). However, even under serum-free conditions, wild-type ABCG1 still resulted in elevated secretion of Aβ40 and Aβ42 (Fig 2B,C), indicating that HDL for ABCG1 increase Aβ levels. As expected, the S220G mutation reduced the cholesterol efflux activity of ABCG1 to that of the vector-only controls (Fig. 2A). Notably, this mutation also reduced Aβ40 and Aβ42 secretion to baseline levels (Fig. 2B,C), demonstrating that the ability of ABCG1 to augment Aβ release requires its enzymatic activity.

ABCG1 also increases sAPPα and sAPPβ secretion. To determine the effect of ABCG1 on release of sAPP species, we next evaluated the levels of sAPPα and sAPPβ in conditioned media from HEK-APPswe cells expressing empty vector or ABCG1. Intriguingly, a significant increase in sAPPα (63% above control, $p=0.020$, N=3), as well as increased sAPPβ (73% above control, $p=0.002$, N=3) was observed in the presence of ABCG1 relative to vector (Fig 3). These observations suggest that ABCG1 may lead to an increase in the availability of APP as a substrate for both α-secretase and β-secretase pathways. APP mRNA levels were unchanged in HEK-APPswe cells expressing vector (APP:GAPDH

ratio = 1.076 ± 0.265, N=3) and ABCG1 (APP:GAPDH ratio = 1.002 ± 0.074, N=3). This observation shows that elevated transcription of APP per se cannot account for the increased production of secretase products, and suggests that the increased catabolism of APP occurs through posttranscriptional mechanisms.

CTFα and CTFβ levels are elevated in cells expressing ABCG1. CTFα and CTFβ fragments were next analysed by Western blot in transfected HEK-APPswe cells. Compared to vector, ABCG1-expressing cells exhibited a significant increase in CTFα ($p=0.0137$, N=3) and CTFβ ($p=0.0066$, N=3), as well as in total APP protein levels. Importantly, the increase in CTF levels remained significant even when corrected for the increase in total APP protein observed in ABCG1-expressing cells (CTFα/APP: $p=0.0136$, N=3; CTFβ/APP: $p=0.0002$, N=3) (Fig 4). Although a proportional increase in CTFα and CTFβ can be caused by decreased γ-secretase activity, our observation that CTFα and CTFβ levels are each increased suggests that expression of ABCG1 promotes increased processing of APP by both the amyloidogenic and nonamyloidogenic pathways. These results are consistent with a model in which expression of ABCG1 leads to increased accumulation of APP holoprotein at sites accessible to secretases.

ABCG1 increases cell surface presentation of APP. Although the precise intracellular sites of APP proteolysis is a subject of considerable debate, it has been reported that all secretases have the ability to cleave APP at the cell surface and/or in early endosomes [15, 58-60]. Because ABCG1 is known to redistribute cholesterol to the plasma membrane [34] and affects both the α-secretase and β-secretase pathways of APP processing, we hypothesized that ABCG1 could increase the proportion of APP at the cell surface that is available for proteolysis by all secretases either at the cell surface or upon endocytosis. Biotinylation assays were therefore used to determine the subcellular distribution of APP in ABCG1-expressing cells. HEK-APPswe cells expressing either murine or human ABCG1 exhibited increased total APP ($p = 0.009$ for murine vs control, $p = 0.003$ for human vs control, N=3), and increased surface APP ($p = 0.01$ for murine, $p = 0.0003$ for human vs control, N=3). Notably, the increase in surface APP was greater than the increase in total APP, resulting in an elevated proportion of total APP at the plasma membrane ($p = 0.097$ for murine vs control, $p = 0.008$ for human vs control, N=3). These results show that the increased cell-surface APP was only partly accounted for by the increase in total APP levels in ABCG1-expressing cells (Fig 5), suggesting that ABCG1 activity preferentially presents APP at the cell surface. Furthermore, continuous sucrose density centrifugation and Western blot analysis showed that an increased proportion of APP colocalized with a plasma membrane marker (β1-integrin) in ABCG1-expressing compared to control HEK-APPswe cells (data not shown). These results from two

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independent methods suggest that ABCG1 activity leads to increased cell surface presentation of APP, which in turn may increase the availability of APP as a secretase substrate.

To test whether increased cell surface APP could be attributed to adherence of secreted APP species to the plasma membrane, HEK293 cells that did not express APPswe were transfected with vector or ABCG1 for 24h to allow for ABCG1-mediated changes in cell surface lipid distribution to occur, then exposed for an additional 24h to conditioned media containing sAPP. Neither vector-transfected nor ABCG1-expressing cells accumulated detectable sAPP (Fig 6), suggesting that ABCG1-mediated changes in membrane composition are not sufficient to attract exogenous sAPP to the cell surface.

ABCG1 is highly expressed in neurons and is overexpressed in DS frontal cortex: Homologous recombination of an IRES-LacZ-Neo-pA cassette into the murine *abcg1* locus allows rapid analysis of ABCG1 expression patterns using β-galactosidase histological staining. Analysis of hemizygous ABCG+/- brains demonstrated that ABCG1 is highly expressed in neurons, with particularly abundant expression in hippocampus where it is found in CA1, CA2, CA3 neurons as well as in the dentate gyrus. ABCG1 is also expressed in all cortical layers, as well as in the striatum and thalamus (Fig 7).

Quantitative RT-PCR was then used to measure the levels of human ABCG1 mRNA from nine post-mortem trisomy 21 cases that ranged in age from 10 to 56 years (Table 1). Each trisomy 21 case was matched by age and sex to a normal control (Table 1). ABCG1 mRNA abundance was also quantified from eight late-onset AD cases that were also matched by age and sex to a normal control (Table 1). ABCG1 mRNA levels in DS frontal cortex were 2.435 +/- 1.43 fold more abundant than in control frontal cortex ($p=0.023$, $N=9$) (Fig 8), clearly demonstrating that inheritance of an extra copy of ABCG1 is associated with elevated ABCG1 mRNA levels in human post-mortem trisomy 21. Notably, no significant difference was observed between ABCG1 mRNA levels in AD brain compared to age- and sex-matched controls ($p=0.911$, $N=8$), showing that the presence of AD neuropathology is not sufficient to upregulate ABCG1 expression. In contrast to ABCG1, we found that APP mRNA levels were not significantly increased in these same trisomy 21 cases relative to controls, although a clear trend toward increased APP expression was observed ($p=0.131$, $N=9$). APP mRNA levels were indistinguishable in AD cases compared to controls ($p=0.846$, $N\geq7$) (Fig 8B).

Western blot analysis of total membrane preparations revealed that ABCG1 protein levels are 2-fold more abundant in DS frontal cortex compared to age-matched controls ($p=0.008$, $N=4$) (Fig 9). In contrast, APP protein levels were not significantly different in these same fractions ($p=0.638$, $N=4$) (Fig 9).

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Aβ and sAPPα levels are elevated in DS cortex. Finally, we determined whether excess ABCG1 in DS is associated with increased processing of APP *in vivo*. First, total Aβ40 and Aβ42 levels were assessed in nine trisomy 21 cases relative to ten controls. As expected, both Aβ40 and Aβ42 were significantly elevated in DS cortex ($p=0.025$ and 0.0001, respectively) (Fig 10A). Next, total APP and sAPP levels were quantified in these same DS and control cases. In whole cortical lysates, total APP protein levels as measured by ELISA were indistinguishable in trisomy 21 cases compared to controls (Fig 10B). The levels of sAPPβ did not differ significantly between DS and control cases, irrespective of whether the sAPPβ measurement was normalized for total APP protein level in each case (Fig 10C,D). In contrast, sAPPα levels tended to be increased in DS cortex compared to controls ($p=0.052$), which was significant when corrected for total APP levels for each case ($p=0.013$) (Fig 10E,F). These results show that expression of excess ABCG1 is associated with elevated Aβ and sAPPα levels in DS brain.

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DISCUSSION

Intracellular cholesterol levels markedly affect APP processing and the subcellular distribution of APP and secretase components [4-14]. However, little is known about how genes that control intracellular lipid distribution may affect APP metabolism. Here we demonstrate that transient expression of the cholesterol transporter ABCG1 affects the proteolytic processing and subcellular distribution of APP *in vitro*. In cultured HEK-APPswe cells, expression of functional ABCG1 elevates Aβ, sAPPα, and sAPPβ secretion and increases the proportion of APP that is present at the cell surface. Because APP and secretases are all membrane bound proteins whose subcellular distribution and activities are highly dependent on cholesterol [6,61-63], it is possible that the activities of ABCG1 in lipid trafficking and efflux may influence the intracellular routing of several gene products involved in APP metabolism. Our observations provide evidence for a novel activity of ABCG1 as a modulator of APP processing and subcellular trafficking, and suggest that ABCG1 may be a key participant in pathways that link cholesterol with APP metabolism.

Observations that increased cholesterol augments Aβ production [4-7], whereas cholesterol depletion stimulates α-secretase activity [8-13] have led to the prediction that genes such as ABCG1 and ABCA1 that promote cholesterol efflux should decrease Aβ levels by the resulting reduction of intracellular sterol content. However, investigations of both ABCG1 and ABCA1 suggest that this prediction may be overly simplistic, and that effects on intracellular cholesterol distribution may be equally important in modulating APP processing than cholesterol levels.

ABCA1 is crucial for the efflux of cholesterol onto lipid-poor apoA-I particles, and other lipid-poor or lipid free apolipoproteins also efficiently accept lipids from ABCA1 [22,64]. Deficiency of ABCA1 leads to accumulation of intracellular cholesterol and a nearly complete lack of circulating HDL [65-67]. Although early *in vitro* studies suggested that ABCA1 influences Aβ production, no consensus was reached on whether ABCA1 increased or decreased Aβ levels [68-70]. Furthermore, four independent groups have now demonstrated *in vivo* that Aβ levels are unaffected by the absence of ABCA1 [24,25,27,28]. We and others have recently shown that ABCA1 influences amyloidogenesis via alterations in apoE metabolism. Mice lacking ABCA1 have severe reductions in apoE levels in the brain, which results from inefficient secretion and lipidation of apoE from glia [23,26]. Importantly, these poorly lipidated apoE particles greatly facilitate the formation of amyloid deposits [24,25,27]. Together, these studies demonstrate that although one function of ABCA1 is to modulate cholesterol levels, ABCA1 does not appear to modulate Aβ production in vivo. Rather, these results suggest that ABCA1 influences amyloid deposition and/or clearance by affecting apoE levels and lipidation.

ABCG1 has also been reported to promote cholesterol efflux, but in contrast to ABCA1, ABCG1 can only transfer cholesterol to lipidated particles such as HDL [32,33]. Here we provide evidence that ABCG1 also affects APP metabolism and that this requires its cholesterol efflux activity. Under our *in vitro* conditions, ABCG1 facilitates APP processing through both the α-secretase and β-secretase pathways, leading to increased secretion of Aβ, sAPPα, and sAPPβ species, which is associated with increased cell-surface presentation of APP. Our results therefore identify a novel function of ABCG1 as modulator of APP trafficking in a cholesterol-dependent manner, and categorize ABCG1 as one of the first genes reported to increase APP presentation at the cell surface.

However, much remains to be learned. For example, it is not yet known whether ABCG1 may also affect the enzymatic activity of secretases that depend on a lipid environment, or whether it may simply influence the interaction of secretases and APP within specific membrane microdomains. We do not know whether ABCG1 may affect the trafficking of APP through anterograde, retrograde, or endocytic pathways. Finally, it is not known whether ABCG1 may contribute to the increased maturation of BACE1 that has recently been identified as a novel mechanism contributing to AD in DS [71]. Understanding the precise mechanisms by which ABCG1 leads to increased cell surface APP and subsequent processing will require evaluating each of these aspects of APP metabolism. Intriguingly, our results differ from a recent report suggesting that ABCG1 suppresses Aβ production in CHO cells in manner unrelated to its cholesterol efflux activity [72], although effects on CTF or sAPP generation and APP subcellular localization were not evaluated. As in the case with ABCA1, *in vivo* studies using mice with a selective increase or deficiency of ABCG1 will be required to evaluate the impact of ABCG1 on the pathogenesis of AD in an appropriate physiological context.

Our findings that DS cortex contains 2-fold more ABCG1 mRNA and protein than age-matched control samples and exhibits increased Aβ and sAPPα levels are consistent with many of our observations in ABCG1-expressing HEK-APPswe cells. Unlike HEK-APPswe cells however, we did not observe increased sAPPβ levels in our cohort of DS post-mortem tissue. There are several potential mechanisms for this discrepancy. For example, the ABCG1 levels achieved in transfected HEK cells may exceed physiological levels. It is also difficult to extrapolate the results of this simple *in vitro* model system to the complexity of the post-mortem DS brain, where increased turnover of sAPPβ relative to sAPPα may be operative. Interestingly, a recent study has reported that CTFα levels decline during aging in DS whereas CTFβ levels increase [73]. It is possible that we were unable to detect these changes given the relatively small number of DS samples of a wide age variation that were examined in this study. Finally, it is important to note that our *in vivo* results are correlative and do not rule out the possibility that other chromosome 21 genes in addition to ABCG1 may also play a role in the development of early AD

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neuropathology in DS subjects. Recently, BACE2, a novel aspartyl protease located on chromosome 21, has been excluded as a potential gene that contributes to the development of AD neuropathology in DS [74]. Future studies will be required to evaluate the impact of selective overexpression or deficiency of ABCG1 on AD neuropathology *in vivo*.

It has long been established that excess APP is required to observe AD neuropathology in DS, as analysis of a single DS subject with partial trisomy 21 that excluded APP found no evidence of amyloid deposition at 78-years of age [50]. Recently, duplication of the APP locus in five independent families was reported to cause autosomal dominant early onset AD with cerebral amyloid angiopathy (CAA) with a mean clinical age of onset of dementia by 52 years in a total of 19 affected individuals [75]. None of these affected individuals had other features of DS such as mental retardation prior to the onset of clinical dementia. However, the relationship between APP gene dose and APP expression in DS is not simple. Increased APP mRNA has been reported in fetal and adult DS brain [76-78], yet several studies have failed to observe significantly elevated APP protein levels in DS compared to control brains [79-81]. Immunohistochemical analysis of postnatal DS brain suggests that the neuronal staining intensity of APP protein increases during aging [80], although a recent study found no association between total APP levels and age [73]. Interestingly, a survey of 41 genes on chromosome 21 demonstrated that APP exhibited the highest degree of inter-individual variability of expression [82], suggesting that individual differences in APP expression levels may also partly account for the varying ability to detect APP overexpression in different DS subjects. In our cohort, we failed to observe significant APP overexpression in DS cortex when evaluated by quantitative RT-PCR, Western blot, or ELISA. Although a clear trend towards increased APP mRNA levels was evident, total APP protein levels were similar in our DS and control cohorts.

In contrast to APP, we consistently observed a robust 2-fold increase in ABCG1 mRNA levels in DS compared to control cortex, consistent with published microarray findings that ABCG1 mRNA levels are increased by 1.43 fold in DS brain and by 1.23 fold in fetal DS cells [78,83]. ABCG1 protein levels were also 2-fold more abundant in DS brain compared to controls. These data clearly show that ABCG1 is overexpressed in DS, which may result in increased processing of APP beyond that accounted for solely by increased APP levels.

This study suggests that the accelerated onset of AD neuropathology in DS may also involve alterations in intracellular lipid trafficking mediated by overexpression of the cholesterol transporter ABCG1. ABCG1 is a half-sized transporter, and the results of several studies including ours have demonstrated that ABCG1 can function as a homodimer. This suggests that inheritance of excess ABCG1 gene dose in DS may be sufficient to elevate functional ABCG1 activity without necessarily invoking a requirement for elevated levels of other half-sized transporters such as ABCG4 that may

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heterodimerize with ABCG1. Using an *in vitro* model system, here we have demonstrated a novel property of ABCG1 in promoting the cell surface presentation of APP and leading to its increased proteolytic processing by secretases. *In vivo*, ABCG1 is highly expressed in neurons, overexpressed in DS brain, and is associated with elevated Aβ and sAPPα levels in human post-mortem tissue. Our observations support the hypothesis that excess ABCG1 in DS may result in an altered distribution of APP that facilitates the generation of neurotoxic Aβ species and accelerates the onset of AD neuropathology in subjects with DS.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1: ABCG1 enhances Aβ production *in vitro.* (A,B) HEK-APPswe cells were transiently transfected with empty vector or ABCG1. Conditioned media was collected at various times, assayed for Aβ40 (A) and Aβ42 (B) by ELISA, and normalized to total cell protein. Data represent the mean and standard deviation of N=2 independent experiments for empty vector and ABCG1. Data were analyzed by unpaired student's t test at each time point, with * representing $p<0.05$, and ** representing $p<0.01$. (C) Western blot shows detection of ABCG1 protein after transfection with ABCG1.

Figure 2: ABCG1 requires cholesterol efflux activity to affect Aβ production. HEK-APPswe cells were transfected with vector, wild-type ABCG1, or ABCG1 containing a S220G mutation in the Walker A domain. (A) Wild-type ABCG1, but not ABCG1 S220G, effluxes cholesterol to exogenous HDL above baseline levels. Data represent the means and standard error from at least two independent cholesterol efflux assays over a 6h period, each measured in triplicate. Wild-type ABCG1, but not ABCG1 S220G, augments Aβ40 (B) and Aβ42 (C) production above baseline levels. Data represent the mean and standard error of at least two independent experiments.

Figure 3: ABCG1 promotes secretion of sAPPα and sAPPβ. HEK-APPswe cells were transfected with ABCG1 or empty vector for 24h, followed by a 6h period of conditioning in fresh media. sAPPα, and sAPPβ levels were evaluated by Western blot analysis (A) of media and normalized to total cellular protein. The graph (B) represents the mean and standard error of a representative experiment of N=3 independent transfections.

Figure 4: ABCG1 enhances α-secretase and β-secretase cleavage of APP. (A) Representative Western blot of total APP as well as CTFα and CTFβ fragments from HEK-APPswe cells transfected with vector standard error of N=3 transfections. A total of three independent rounds of transfections, each at least in triplicate, were performed

Figure 5: ABCG1 increases cell surface presentation of APP. (A) Representative Western blot of HEK-APPswe cells transiently transfected with empty vector, murine ABCG1, or human ABCG1, and treated with sulfo-NHS-biotin followed by precipitation of biotinylated cell surface proteins using Strepavidin agarose beads. The levels of total and cell-surface APP are shown and normalized to actin levels as an internal control. (B) Quantitation of APP distribution. The graph represents the means and standard error

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of a representative round of three independent transfections, analysed by Student's t-test. A total of N=3 rounds of at least triplicate independent transfections were performed.

Figure 6: ABCG1 does not increase cellular adherence of exogenous sAPP. (A) Conditioned media and cell lysates were prepared from HEK-APPswe and HEK293 cells (Input APP and HEK). HEK293 cells were transfected with vector or ABCG1 and exposed to conditioned media from HEK293 cells (HEK CM), or to conditioned media from HEK-APPswe cells (APP CM) for 24h, after which media and cell lysates were immunoblotted for APP and GAPDH. The upper panel (Media sAPP) shows the input levels of sAPP in the conditioned media at the beginning of the experiment, and demonstrates no loss of input signal after 24h of incubation on transfected cells. The middle panel (Cellular APP) shows the levels of cell-associated APP in HEK-APPswe and HEK293 cells (Input lanes), and in HEK293 cells exposed to HEK293 CM or APP CM, using 75 μg protein per lane. The lower panel shows GAPDH as an internal loading control.

Figure 7: ABCG1 is highly expressed in neurons. LacZ staining of ABCG1 heterozygous (a,c,e,g,h,i) and wild-type (WT) (b,d,f) mice. Coronal sections are shown at 2.5x (a,b), 10x (c-f), and 40x (g-i) magnification. Strong lacZ staining, indicative of ABCG1 expression, is observed in hippocampus (a, c), all cortical layers (a,e,g,h,i), striatum and thalamus (a).

Figure 8: ABCG1 mRNA levels are significantly elevated in DS cortex. QRT-PCR was used to determine ABCG1 (A) and APP (B) mRNA levels from eight trisomy 21 cases (black bars) matched to eight age and sex-matched controls (clear bars), as well as eight AD cases (grey bars) matched to eight age and sex-matched controls (hatched bars). Data represent the means and standard error of each case measured in duplicate for the DS and matched controls, and in triplicate for the AD cases and matched controls, analysed by Student's unpaired t-test.

Figure 9: ABCG1 protein levels are significantly elevated in DS cortex. (A) Western blots of total membrane fractions from four age-matched control and trisomy 21 cases. Blots were probed sequentially for ABCG1, APP, and NaK-ATPase that served as an internal loading control. (B) Relative ABCG1/NaK-ATPase and APP/NaK-ATPase values are shown, with the values in control fractions set to 1. Data represent the means and standard error of each case assayed in duplicate and analysed by Student's unpaired t-test.

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Figure 10: Aβ and sAPPα levels are elevated in DS cortex. (A) Aβ40 and Aβ42 levels were determined by ELISA in cortex (D) of nine trisomy 21 cases (DS) and ten controls (Con). Data represent the mean and standard error. (B) Total APP levels were quantified by ELISA from these same trisomy 21 cases and controls. (C, D) sAPPβ and (E, F) sAPPα were determined by ELISA from these same trisomy 21 cases and controls. Data is presented as the scatter of sAPPβ and sAPPα levels expressed before (C, E) and after (D, F) normalization to APP levels in each brain sample, with the line representing the mean.

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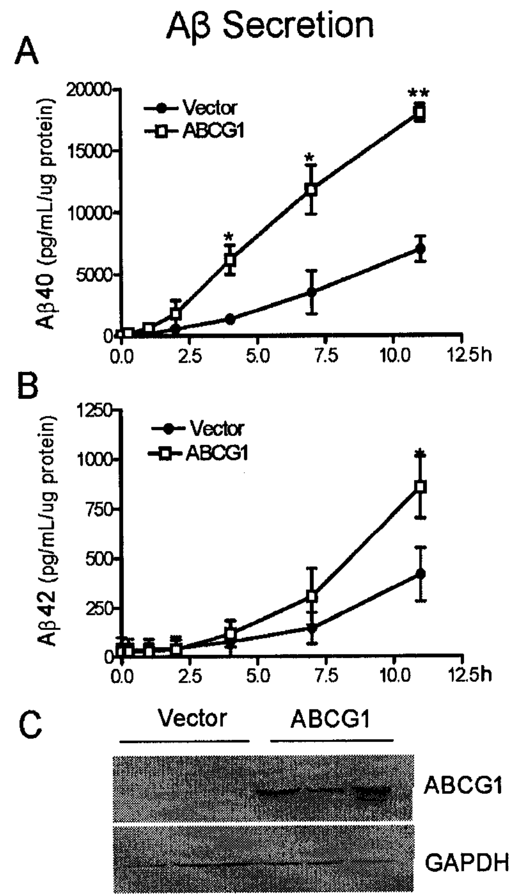
Fig 1



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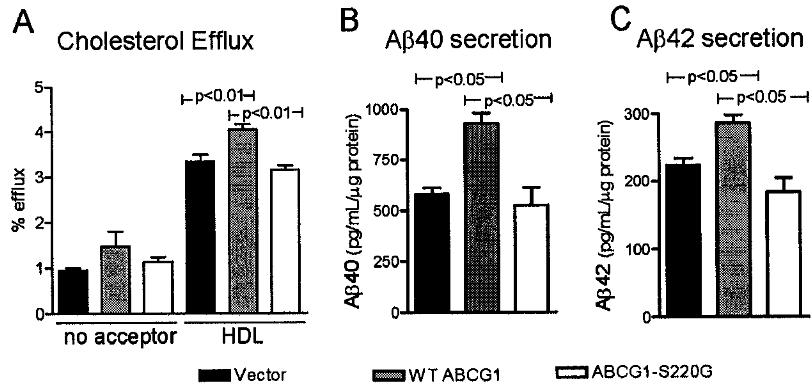

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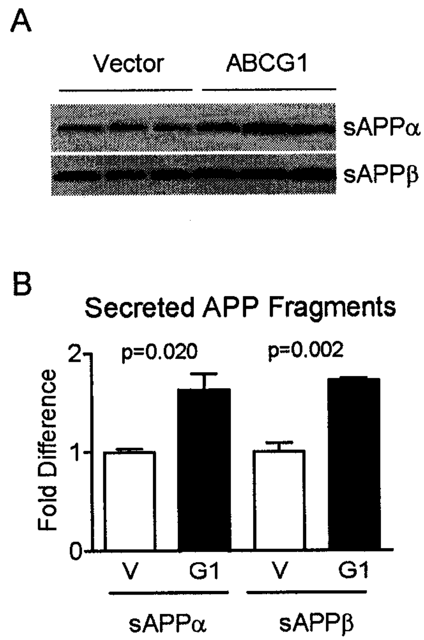

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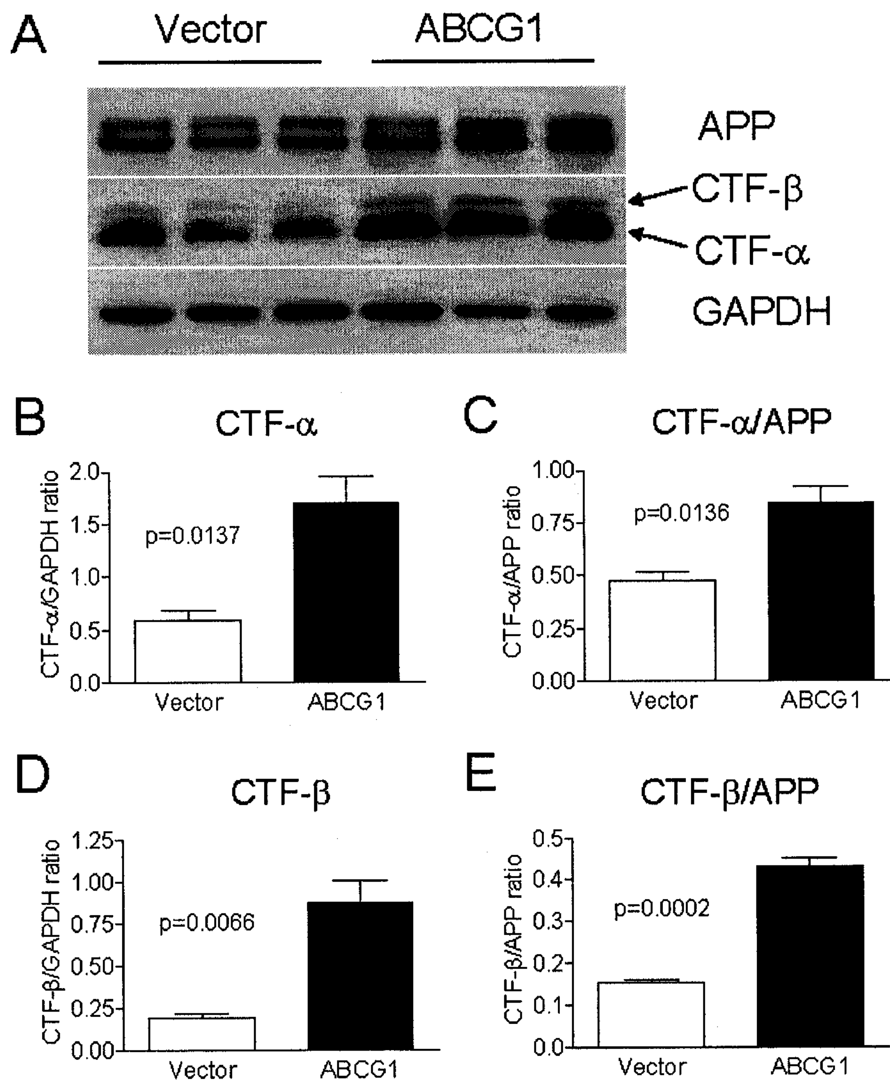

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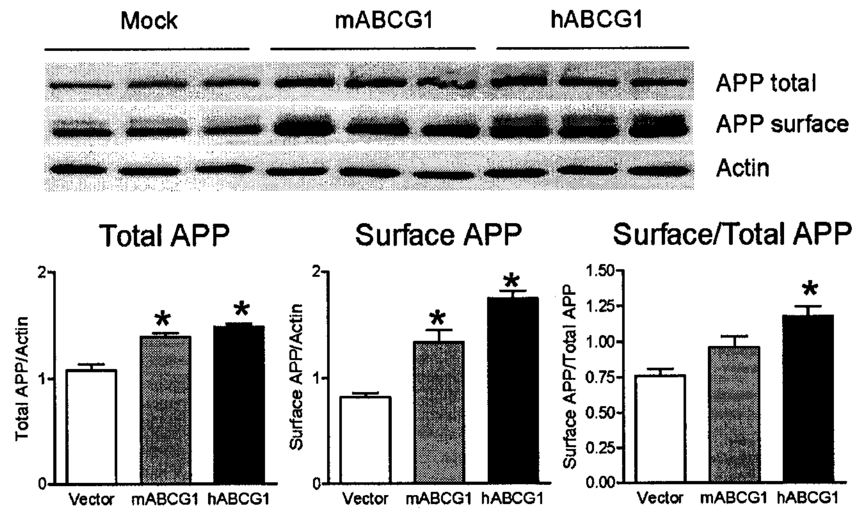

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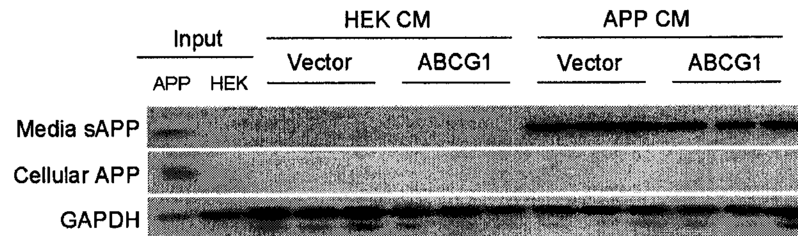

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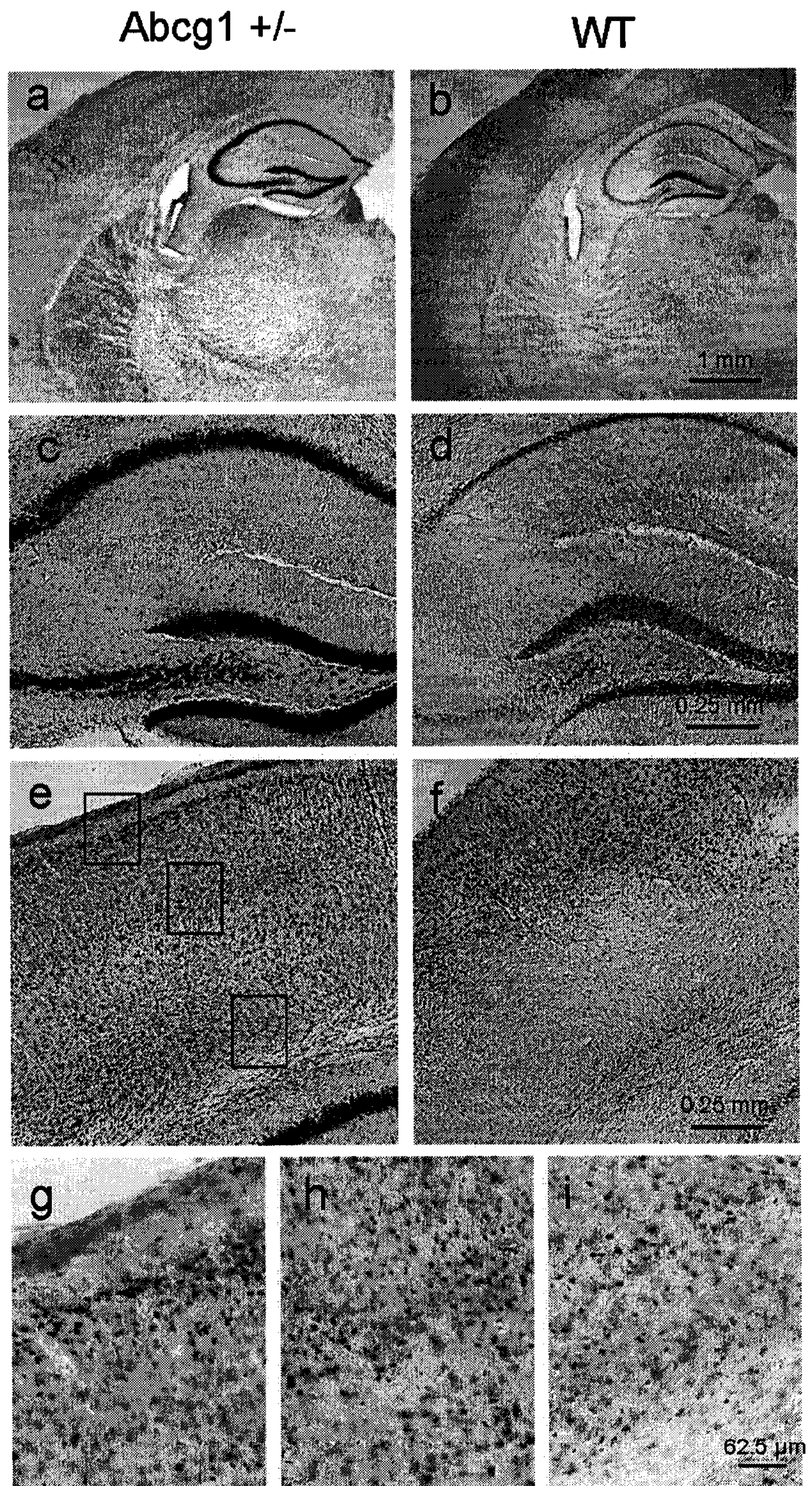

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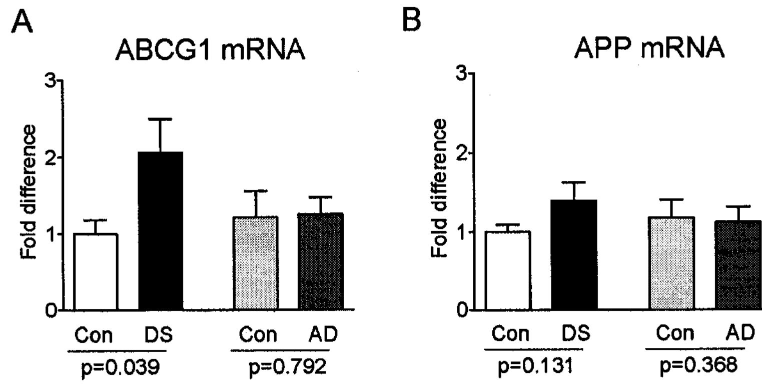

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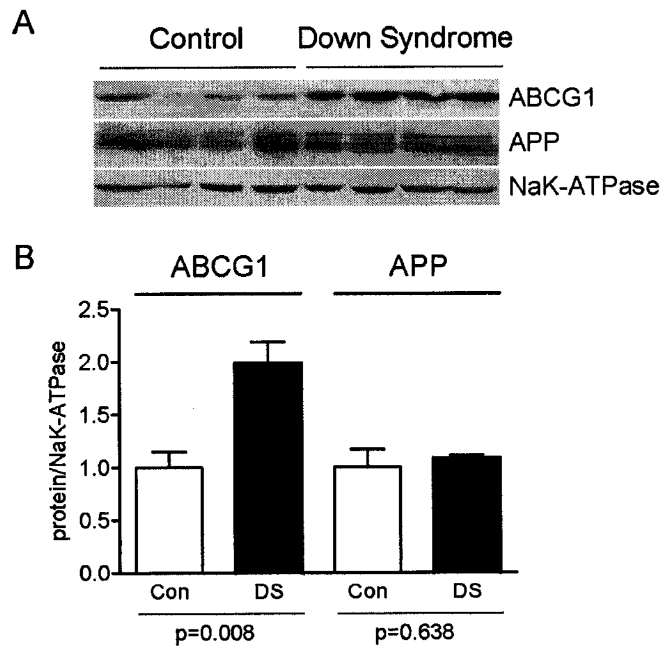

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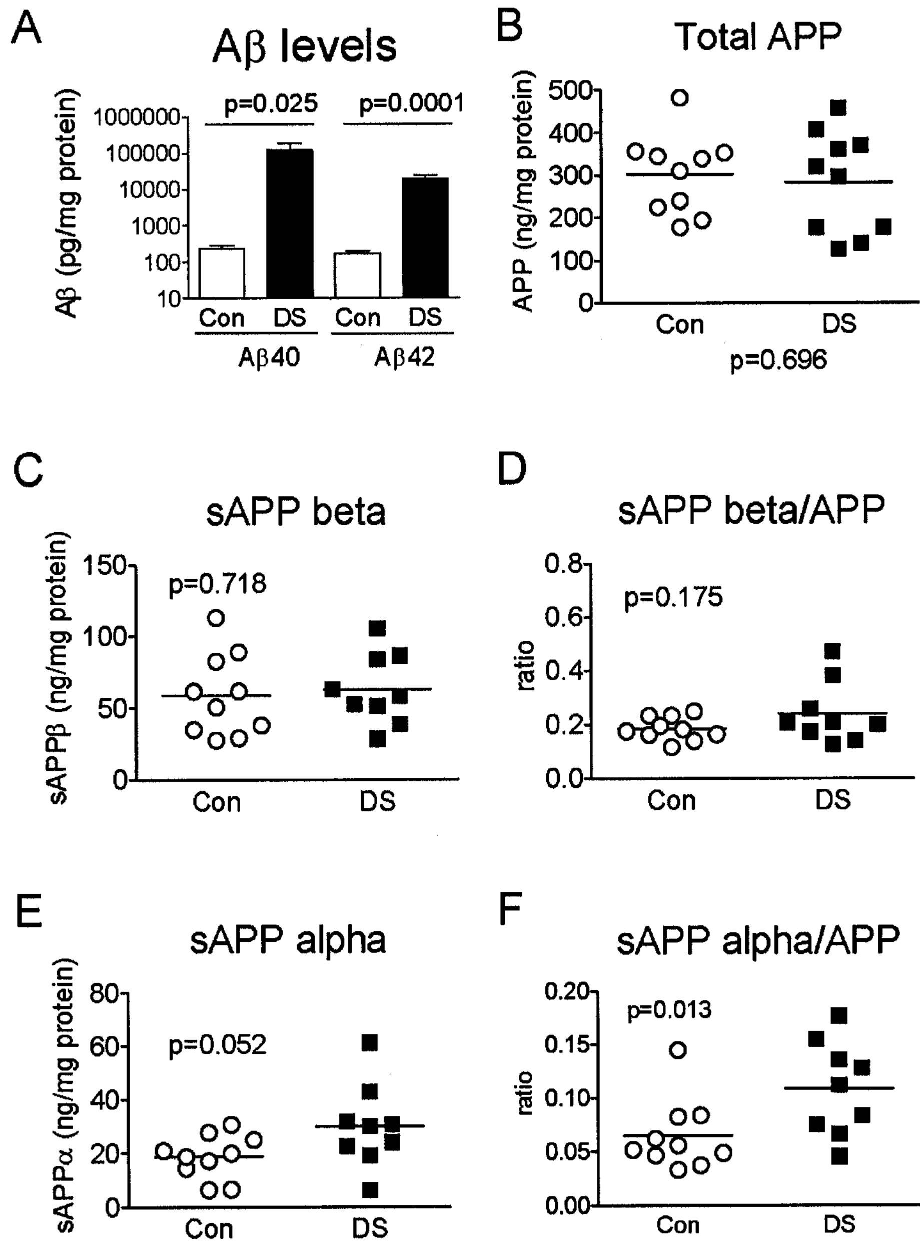


Table 1: Human Tissues

Type	Sample ID	Age	Sex	Ethnicity	PMI	Type	Sample ID	Age	Sex	Ethnicity	PMI
Control	1793	11	M	AA	19	DS	1267	10	M	AA	15
Control	1037	19	M	C	11	DS	1960	19	M	AA	14
Control	777	22	M	AA	4	DS	707	22	M	AA	15
Control	1441	31	M	C	28	DS	753	23	M	C	24
Control	1134	41	M	C	15	DS	1258	44	F	C	13
Control	1454	47	F	C	24	DS	4659	46	F	C	7
Control	4640	47	F	C	5	DS	3233	47	F	N/A	20
Control	1113	56	M	C	17	DS	3572	51	M	C	20
Control	1206	57	M	C	16	DS	1623	56	M	C	7
Control	1444	79	F	C	14	AD	1252	78	M	C	9
Control	4534	70	M	AA	28	AD	1172	79	F	C	12
Control	4546	86	F	C	22	AD	1312	87	F	C	7
Control	1113	56	M	C	17	AD	1562	83	F	C	6
Control	1206	57	M	C	16	AD	1630	84	F	C	5
Control	1441	51	M	C	28	AD	200	68	M	ND	5
Control	1454	47	F	C	24	AD	429	85	F	ND	16
Control	1134	41	M	C	15	AD	413	73	F	ND	24

Appendix 2: Copy of UBC Animal Care Committee Certificates

The University of British Columbia

ANIMAL CARE CERTIFICATE
Breeding Programs

PROTOCOL NUMBER: **A02-0268**

INVESTIGATOR OR COURSE DIRECTOR: **Wellington, C.**

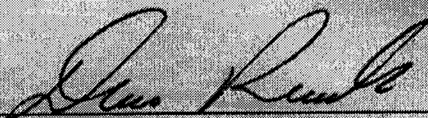
DEPARTMENT: **Pathology & Laboratory Med**

PROJECT TITLE: **BREEDING: The role of the cholesterol transporters ABCA1 and ABCG1 in brain, peripheral, and reproductive systems**

ANIMALS: **Mice 500**

APPROVAL DATE: **04-01-19**

The Animal Care Committee has examined and approved the use of animals for the above breeding program, and have been given an assurance that the animals involved will be cared for in accordance with the principles contained in Care of Experimental Animals - A Guide for Canada, published by the Canadian Council on Animal Care.



Approval of the UBC Committee on Animal Care by one of:
Dr. W.K. Milsom, Chair
Dr. J. Love, Director, Animal Care Centre
M. L. Macdonald, Manager

This certificate is valid for one year from the above approval date provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
110, 6190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111 FAX: 604-822-5093

The University of British Columbia

ANIMAL CARE CERTIFICATE

PROTOCOL NUMBER: **A02-0267**

INVESTIGATOR OR COURSE DIRECTOR: **Wellington, C.**

DEPARTMENT: **CMMT**

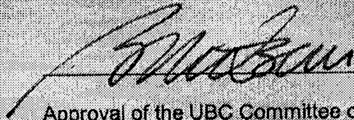
PROJECT OR COURSE TITLE: **ABCA1, cholesterol transport, and Alzheimer's Disease**

ANIMALS: **Mice 1200**

START DATE: **02-10-31** APPROVAL DATE: **04-01-19**

FUNDING AGENCY: **Canadian Institutes of Health Research**

The Animal Care Committee has examined and approved the use of animals for the above experimental project or teaching course, and have been given an assurance that the animals involved will be cared for in accordance with the principles contained in Care of Experimental Animals - A Guide for Canada, published by the Canadian Council on Animal Care.



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Ms. L. Macdonald, Manager, Animal Care Committee

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