MODIFIERS OF BETA-AMYLOID METABOLISM AND DEPOSITION IN MOUSE MODELS OF ALZHEIMER’S DISEASE

by

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*We also certify that written approval has been obtained for any proprietary material contained therein.
This thesis is dedicated to my children,
the greatest accomplishments of my life.
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Abstract

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Alzheimer’s disease (AD) is a progressive form of dementia that is definitively diagnosed postmortem by the presence of beta-amyloid (Aβ) deposition. Aβ is the proteolytic processing product of the amyloid precursor protein (APP). While genetic evidence supports APP as disease causative in some cases of AD, it accounts for only 1% of the predicted 79% heritability of the disease. The majority of genetic factors involved in AD are largely unknown, as is how they interact with the environmental factors responsible for the disease. Although genetic screens in humans have been unsuccessful in reproducibly identifying genetic and environmental risk factors for AD, the congenic R1.40 mouse model provides a reliable method to identify genetic risk factors for disease. The present study examines the effect of genetic background on Aβ levels throughout life. The C57BL/6J strain has high levels of Aβ early in life, corresponding to Aβ deposition at 13 months of age. In contrast, the DBA/2J and 129S1/SvImJ strains have reduced levels of Aβ early in life, with delayed deposition until 24 months of age. Finally, the A/J strain has Aβ levels significantly higher than those in the C57BL/6J strain but never deposits. Unique genetic mapping resources available for these strains, including chromosome
substitution strains (A/J) and congenic lines (DBA/2J) were used to identify specific loci responsible for alterations in Aβ metabolism. The regions containing genetic modifiers of Aβ deposition were narrowed to chromosome 11 in the A/J strain, chromosome 1 in the DBA/2J strain and have identified a gene (C5) responsible for reduced Aβ deposition on chromosome 2 in the DBA2/J strain. This thesis also examines the complex interaction between genes and environment, showing that genetic background can affect susceptibility to postulated environmental modifiers of Alzheimer’s disease. Hemizygous R1.40 animals on three genetic backgrounds, DBA/2J, C57BL/6J and 129S1/SvImJ were fed high-fat/high-cholesterol (HF/HC) or low-fat/low-cholesterol (LF/LC) diets for 8 weeks. Despite all strains being exposed to a HF/HC diet, only the C57BL/6J strain had alterations in Aβ levels, which appeared to be due to diet-induced strain-specific differential gene expression.
Chapter 1: Introduction and Research Aims
Introduction to Alzheimer’s Disease

Alzheimer’s disease (AD) was first described over 100 years ago, by Dr. Alois Alzheimer, in his 51 year old patient, Auguste D. She presented with memory loss and temporal and spatial disorientation that rapidly progressed over a period of 4½ years, culminating in a complete inability to engage the world and ultimately death (Alzheimer et al., 1995; Maurer et al., 1997). Upon postmortem analysis, Dr. Alzheimer observed the presence of several pathological alterations (senile plaques and neurofibrillary tangles) that are currently accepted as diagnostic hallmarks of Alzheimer’s disease (Small and Cappai, 2006).

While AD was originally considered a relatively rare condition, increased longevity experienced in the Western world has resulted in an increased prevalence of the disease. Recent figures indicate that ~35 million people are currently afflicted with AD worldwide with the current costs of caring for these individuals estimated to be $315 billion per year. The number of individuals with the disease is projected to double every 20 years, with a projected 115.4 million people affected in 2050, resulting in a devastating impact on the world economies and health care systems (International, 2010).

Since age is the greatest risk factor for AD, the ability to delay the disease by just 5 years is projected to potentially cut the incidence and cost of the disease in half (Brookmeyer et al., 1998). While finding a cure for the disease remains the gold standard, finding therapeutic treatments that can delay its onset and progression are equally important and perhaps more realistic. Identification
of such potential therapies requires a better understand of the genetic and environmental pathways underlying the disease.

**Alzheimer’s Disease Hallmarks**

AD has several clinical and pathological hallmarks. Clinically, it is defined as a progressive form of dementia. It begins as mild cognitive impairment (MCI). In this stage, individuals are relatively normal in other cognitive domains and have intact activities of daily living with a slight cognitive impairment of insufficient severity to constitute dementia (Petersen et al., 2001). The disease then progresses over 5 to 8 years. Approximately 12% of individuals with MCI progress to AD each year, by 6 years 80% of MCI patients have converted to dementia (Petersen and Morris, 2003). Over time, patients develop personality changes, behavioral disturbances, and confusion. Eventually the disease affects the ability to perform basic functions such as eating and drinking and ultimately results in death (Petersen, 2004).

There are also several pathological hallmarks intimately associated with AD (NIA, 1997). The first is intracellular aggregates of the hyperphosphorylated microtubule associated protein tau within neurons, termed neurofibrillary tangles (NFTs). While part of AD, NFTs are also associated with other neurodegenerative diseases, including frontotemporal dementia, frontotemporal lobar degeneration (Pick’s disease), argyrophilic grain disease, progressive supranuclear palsy, and corticobasal degeneration. The second pathological hallmark of AD is senile plaques, unique to AD, they are dense extracellular
aggregates composed of the beta-amyloid (Aβ) peptide, a 40-42 amino acid peptide derived from the amyloid precursor protein (APP). The exact relationship between these two major neuropathologies observed in AD and the role they play in the clinical phenotypes observed in the disease remains quite unclear.

**Alzheimer's Disease Epidemiology**

The major known risk factors for AD include age as well as both environmental and genetic factors.

**Age**

Age is the single greatest risk factor for AD. The incidence of AD doubles every 5 years after age 65 (Aging, 2008). Studies have suggested ~40% to 50% of individuals over 85 years of age have the disease (Evans et al., 1989).

**Environment**

Epidemiological studies have investigated the roles of a large number of environmental risk factors in the development of AD. However, relatively few of these environmental risk factors have been replicated in a large number of studies, including the protective effects of education and exercise and the detrimental effects of repeated, traumatic brain injury and hypercholesterolemic diet.
**Education**

Multiple studies have demonstrated that increased education, higher occupational attainment, and participation in intellectual aspects of daily life appear to be protective against AD and age-related cognitive decline (Butler et al., 1996; Katzman, 1993; Ott et al., 1999; Snowdon et al., 1989; Snowdon et al., 1996). Based upon these findings, the cognitive reserve hypothesis was put forward which stipulates that these educational activities result in functionally more efficient cognitive networks, providing cognitive reserves that can tolerate more AD pathology before resulting in clinical manifestations of dementia (Scarmeas and Stern, 2004).

**Exercise**

Several different studies have demonstrated that exercise may be protective against the development of AD and several hypotheses have been put forward to explain this association (Larson et al., 2006; Laurin et al., 2001; Radak et al., 2010; Wang et al., 2006). First, exercise is known to reduce stress and relieve depression, which have also been associated with AD (Salmon, 2001; Simard et al., 2009). Second, there is considerable evidence that free radical damage to RNA, proteins, and lipids occurs within the AD brain and it has been demonstrated that increased physical activity is associated with enhanced oxidative repair mechanisms, leading to the hypothesis that exercise could reduce oxidative damage thus resulting in protective effects in the AD brain (Radak et al., 2006; Smith et al., 1996). Third, physical activity stimulates
neurogenesis, potentially resulting in enhanced memory and brain plasticity and it has been hypothesized that this could counteract the deleterious affects of AD on cognitive functions (Nagahara et al., 2009; Radak et al., 2006). Finally, several studies demonstrate that exercise enhances Aβ degradation, potentially through increase in the activity of Aβ degrading enzymes, including insulin degrading enzyme (IDE) and membrane metalloendopeptidase (MME) (Lazarov et al., 2005).

*Traumatic Brian Injury*

Multiple independent studies have implicated traumatic brain injury (TBI) as a major risk factor for AD (French et al., 1985; Graves et al., 1990; McKenzie et al., 1994; Mortimer et al., 1985; Mortimer et al., 1991; Rasmusson et al., 1995; Rudelli et al., 1982; Salib and Hillier, 1997; van Duijn et al., 1992). Based on these studies, it has been estimated that moderate head injury doubles an individual’s risk of dementia and that severe head injury quadruples that risk (Plassman et al., 2000). Furthermore, retrospective studies in humans have shown that one third of individuals with a fatal TBI have Aβ deposits in the brain, significantly higher than control populations (Clinton et al., 1991; Gentleman et al., 1997; Roberts et al., 1990; Roberts et al, 1991; Roberts et al., 1994). The increased risk of AD associated with head trauma has been supported experimentally in a pig model of traumatic axonal injury, which induces Aβ plaque pathology (Smith et al., 1999). One potential mechanism via which TBI has been hypothesized to elevate AD risk is through enhanced expression of the
amyloid precursor protein (APP) as well as elevated proteolytic processing of APP into Aβ within neurons that ultimately results in elevated extracellular Aβ deposition (Chen et al., 2004).

**Diet**

There is conflicting evidence as to whether a western, high-fat/high-cholesterol diet may contribute to AD risk. Several studies have failed to find evidence for a link between cholesterol and AD. For example, a recently published study in Swedish women (The Prospective Population Study of Women) enrolled non-demented patients in their 3rd-6th decade of life and assessed them periodically over the next 30 years for cognition and cholesterol levels. The study found that elevated midlife cholesterol levels were not associated with increased AD risk (Mielke et al., 2010). Similarly, the Honolulu-Asia Aging Study examined Japanese American men over a 26 year period for cholesterol levels and dementia and found no association between midlife cholesterol levels and AD (Stewart et al., 2007). In addition, the Framingham Study enrolled individuals from Framingham Massachusetts in 1950, examined the levels of cholesterol periodically over a period of 38 years, and assessed patients for dementia prior to the conclusion of the study. Overall, the study found no association between cholesterol levels and AD (Tan et al., 2003).

On the other hand, multiple other studies have documented evidence for a relationship between cholesterol levels and AD risk. For example, the Kaiser Permanente Northern California Study assessed individuals at age 40-45 years
and then 30 years later looked for evidence of AD. This study found that midlife serum total cholesterol was associated with increased risk of AD (Solomon et al., 2009). Similarly, a study conducted in eastern Finland over 21 years examined the association between cholesterol levels and AD risk. They found that midlife total serum cholesterol levels were a risk factor for AD and verified a previous finding in another Finnish population (Kivipelto et al., 2002; Notkola et al., 1998). Finally, in an effort to overall assess the link between cholesterol levels and AD risk, a meta-analysis of 18 prospective studies was performed and suggested that indeed high cholesterol levels in mid-life increases the risk for AD late in life (Anstey et al., 2008).

Likewise, there are conflicting findings on the ability of cholesterol lowering drugs, i.e., statins, to reduce AD risk. Multiple, independent retrospective studies have observed a lower incidence of AD in individuals taking statins (Dufouil et al., 2005; Hajjar et al., 2002; Jick et al., 2000; Rockwood et al., 2002; Wolozin et al., 2000; Zamrini et al., 2004). However, the prospective Cardiovascular Health Cognition Study, an ancillary study of the Cardiovascular Health Study, examined 2798 individuals from North Carolina, California, Maryland and Pennsylvania for risk of dementia based on statin exposure. The study found that statin use was not associated with the risk of dementia (Rea et al., 2005).

When looking at the effect of statins as a therapeutic agent, the results were again mixed. The Alzheimer's Disease Cholesterol-Lowering Treatment trial consisted of 98 mild to moderate AD patients from Sun City, Arizona that examined the effects of a 6 month statin treatment. This study observed
improved cognition in the individuals taking statins, with the greatest benefit to those with the highest cholesterol levels (Sparks et al., 2006). In contrast, the LEADe (Lipitor's Effect in Alzheimer's Dementia) study, which enrolled 640 mild to moderate AD patients from 10 countries including the United States, Germany, Canada, the United Kingdom, Australia, Spain, South Africa, Sweden, Austria and Denmark and examined the effect of a 72 week statin treatment, failed to observe any significant alterations in cognition (Feldman et al., 2010). A Cochrane review, a systematic review of primary research to assess the clinical efficacy of statins in the treatment of dementia, found insufficient evidence to recommend statins for the treatment of AD (McGuinness et al., 2010).

While it remains clear that various environmental factors, including age, education, exercise and diet can likely influence AD risk, the exact role these risk factors play in disease onset and progression as well as the potential mechanisms of action remain to be identified. By contrast, various genetic studies of AD have provided the most valuable insights into the underlying biological mechanisms of AD.

Genetics

Other than age, family history is the most significant risk factor for AD. While numerous studies have been conducted to determine the contribution of genetic factors in AD pathogenesis, there overall was a lack of consensus in heritability estimates due to small sample size (case studies ranging in size from one pair of twins to 94 individuals with AD), biased sample selection (studies that
enrolled volunteers rather than examining a cross-section of the population), or
diagnoses based on registry information (AD was determined from files instead
of direct clinical evaluation of individuals). However, in 2006 a study was
published that carefully established the heritability of AD. This study used the
Swedish Twin Registry, which includes all twins born in Sweden since 1886
totaling more than 170,000 twins (Lichtenstein et al., 2006). The study included
five groups: monozygotic (MZ, that share all genes) and dizygotic (DZ, that
share on average 50% of segregating genes) twins of both gender and unlike-
sex pairs. All twins in the registry were examined for cognitive dysfunction at age
65. Through this process, 11,884 twin pairs (428 MZ men, 655 MZ women, 679
DZ men, 1009 DZ women, 1454 unlike-sex twins) and 392 pairs with one or both
member with AD were identified. The study found higher concordance (both
twins having the disorder) of AD in monozygotic twins compared with their
dizygotic counterparts. Based upon statistical arguments, it estimated heritability
of AD to be 79%, with the rest of disease risk due to environmental factors
(Figure 1-1). The study also demonstrated a genetic influence on the timing of
the disease (Gatz et al., 2006).

Based upon genetic criteria, it has been determined that AD can be
classified as one of two types; early onset familial AD (EOFAD) and late onset
familial AD (LOFAD). EOFAD is defined as an age of onset of <60 years and
generally follows an autosomal dominant mode of inheritance, while LOFAD has
an age of onset of >60 years and has a complex multi-modal mode of
inheritance.
Although Dr. Alzheimer first described the clinical and pathological hallmarks of AD in 1906, the genetic basis of the disease wasn’t established until the later part of the 20th century. In the 1950s, it was discovered that Down syndrome (DS) was caused by an extra copy of chromosome 21 (Lejeune et al., 1959). Early observations that individuals with DS presented with symptoms of AD in their fourth decade of life and had pathological hallmarks of AD (senile plaques and NFTs) led investigators to examine chromosome 21 for association with EOFAD once the molecular tools for genetic linkage became available in the late 1970s and early 1980s. Subsequent genetic linkage analysis identified several EOFAD pedigrees that exhibited linkage to human chromosome 21 (Prasher et al., 1998; St George-Hyslop et al., 1987). Around the same time, Aβ was identified as the primary component of senile plaques (Glenner and Wong, 1984). It was not long before scientists discovered that Aβ is derived from APP, a larger precursor protein and that the gene encoding APP was located on chromosome 21 (Goldgaber et al., 1987; Tanzi et al., 1987). To date, 32 mutations in APP have been identified in 89 EOFAD families (http://www.molgen.vib-ua.be/ADMutations/). The vast majority of these mutations are autosomal dominant missense mutations, while two recessive mutations have been identified, one of which is the result of a microdeletion (Bettens et al., 2010; Di Fede et al., 2009; Tomiyama et al., 2008). In addition, recent studies have identified cases of EOFAD caused by microduplications of APP (Blom et al., 2008; Cabrejo et al., 2006; Campion and Hannequin, 2006;
Guyant-Marechal et al., 2008; Kasuga et al., 2009; Rovelet-Lecrux et al., 2007; Rovelet-Lecrux et al., 2006; Sleegers et al., 2006). Taken together, these genetic studies demonstrated that either overexpression (in the case of DS and APP microduplication) or missense mutations in APP could both cause AD.

However, not all EOFAD cases exhibited linkage to APP and chromosome 21. Additional genetic linkage analysis revealed associations to presenilin 1 (PSEN1) on chromosome 14 and presenilin 2 (PSEN2) on chromosome 1 in multiple EOFAD families. Notably, both PSEN1 and PSEN2 have been implicated in the proteolytic processing of APP into Aβ (described below). To date, 182 disease-causing mutations in PSEN1 have been identified in 401 EOFAD families and 14 disease-causing mutations in PSEN2 have been identified in 23 EOFAD families (http://www.molgen.vib-ua.be/ADMutations/). The vast majority of these mutations are autosomal dominant missense mutations scattered throughout the protein, although several small deletions and insertions have also been identified (Bettens et al., 2010). Taken together the studies of EOFAD have implicated the production (APP duplication) and proteolytic processing (APP missense mutations and PSEN1/PSEN2 mutations) of APP into Aβ (as described in detail below) as disease causitive. However, EOFAD accounts for <1% of all AD.

**LOFAD**

The overwhelming majority of AD cases are LOFAD. Numerous genetic studies have revealed a complex mode of inheritance for LOFAD, with the
presence of a relatively large number of independent genes conferring small individual effects (Bertram et al., 2008; Coon et al., 2007). Unlike genes associated with EOFAD that are disease causing, the genes associated with LOFAD alter disease risk. Because of difficulties in identifying genes associated with modest effects on AD risk, it has been estimated that most of the genes responsible (70%-80%) have yet to be identified (Gatz et al., 2006).

In general, two approaches have been used to identify LOFAD genes: family studies, in which LOFAD is inherited through multiple generations, and case-control association studies, in which hundreds to thousands of AD cases and controls from similar populations are examined for genetic factors associated with risk of disease. Family studies have been successful in identifying risk factors for LOFAD. In fact, early genetic linkage analysis revealed an association to chromosomal 19, which was subsequently localized to the Apolipoprotein E (APOE) gene, which is one of the strongest genetic risk factors identified for common diseases with a complex mode of inheritance (Pericak-Vance et al., 1991).

While there have been genetic associations with a handful of genes, the only one consistently identified is the APOE gene (Bertram et al., 2007). It is unlikely there will be another gene identified with an effect size as large as APOE. The gene has three alleles: ε2, ε3, ε4. The presence of the ε4 allele presents a dose-dependant increase in AD risk (approximately four-fold) compared with noncarriers (Corder et al., 1993; Strittmatter et al., 1993). The rarer ε2 allele appears to be protective (Corder et al., 1994). APOE is a lipid
transport protein that binds to Aβ and co-localizes with senile plaques in the AD brain (Shao et al., 1997). Experimental evidence suggests APOE likely plays a critical role in regulating Aβ peptide levels through facilitation of Aβ deposition and clearance (Holtzman, 2001; Jiang et al., 2008; Kamboh, 2004).

Although multiple candidate genes have been tested using case-control studies, none of these genes has been robustly replicated and universally confirmed. Several explanations for the difficulty in identifying these genes using case-control studies have been proposed. First, there is a need for very large sample sizes, in order to ensure that a small effect size will be observed and statistically verified. Second, as mentioned above, there are many environmental modifiers of disease phenotypes that could make replication difficult if not impossible. Third, there is considerable phenotypic heterogeneity: each individual with the disease has a unique combination of disease characteristics, including variation in the age of onset and progression of the disease. This makes it difficult to determine if an individual will go onto develop AD, particularly in the early stages of the disease (Lopez-Aberola et al., 1997). Fourth, there is also genetic heterogeneity: the presence of multiple genes responsible for AD that may all together influence the risk of developing AD. Fifth, there is also evidence for epistasis, in which gene-gene interactions result in altered disease risk. Sixth, there are significant issues with population stratification, in which allele frequencies in a population due to ancestry can bias the results of the genetic study.
Two types of case-control association studies are the candidate gene approach and the genome-wide association studies (GWAS). Numerous association studies have tried to identify genes responsible for LOFAD. Almost 700 genes have been assessed genetically over the past 30 years using a candidate gene approach; however, only a few genes have shown significant risk and been replicated in multiple independent studies (Bertram and Tanzi, 2008). Since the candidate gene approach is hypothesis driven or based on gene function or position, it can only identify genes previously associated with the disease. This is remedied by GWAS, in which millions of single nucleotide polymorphisms (SNPs) interspersed at high density throughout the chromosome are interrogated to provide genome-wide associations. Although GWAS have been successful at identifying novel genes and genetic loci as well as providing insight into biological pathways of human complex diseases (Table 1-1), there are problems and limitations to these studies as well. First, the majority of risk alleles identified by GWAS are common and have a large effect size. However, common SNPs account for only a small portion of total inherited risk of complex disease. Because most arrays focus on common SNPs, GWASs are able to identify only a subset of total genetic variants. These GWAS SNP arrays often overlook lower frequency SNPs; SNPs located in the intron, intergenic, or gene desert regions that coincide with regulatory elements such as enhancers, insulators, transcription factor binding sites, and sequence encoding microRNAs; non-SNP variants and rare SNPs/mutations. Also, the genetic association identified by the GWAS generally does not identify the actual disease-causing
variant, but instead identify a SNP or series of SNPs that associates with the disease phenotype. As a result of these issues, GWAS studies have often exhibited a large false-negative rate and lead to a significant amount of missing heritability (Bertram et al., 2010; Goldstein, 2009; Hardy and Singleton, 2009; Hirschhorn, 2009; Kraft and Hunter, 2009; Ku et al., 2010). Finally, regions identified by GWAS need to be followed up with targeted sequencing and biochemical studies to identify and confirm the sequence variants responsible for AD risk and characterize the presumed molecular effects. At present, there is considerable interest in utilizing whole genome sequencing or other methods to identify rare variants for use in GWASs.

As has been described above, no method is perfect in identifying all the genetic risk factors for AD. Another approach in identifying LOFAD genes in humans is meta-analysis, which synthesizes all of the published genetic data for each polymorphism within a given gene and generates a summary risk estimate (odds ratio [OR]) that takes into account inter- and intra-study variations (Bertram et al., 2007). Whereas GWASs have limited statistical power to detect common alleles with small effect sizes as well as rare variants with larger effect sizes, combining data sets in a meta-analysis enhances statistical power. Thus far, data and results from more than 1000 genetic studies have been analyzed via meta-analysis (Bertram and Tanzi, 2008). At the time this thesis was written, the top 10 genes identified by meta-analysis as having a significant risk effect are APOE, CLU, PICALM, EXOC3L2, BIN1, CR1, SORL1, GWA_14q32.13, TNK1,
and *IL8* (Table 1-2). Numerous studies are currently underway to confirm and/or extend these findings in additional populations.

In summary, a majority of the genes thus far identified in studies of both EOFAD and LOFAD implicated alterations in the production and processing of APP into Aβ as well as the clearance and deposition of Aβ into senile plaques.

**APP, Abeta and the Amyloid Hypothesis**

APP Processing

The *amyloid precursor protein* (*APP*) gene, consisting of ~200 kb and 18 exons, encodes a ubiquitously expressed type 1 transmembrane protein (Goldgaber et al., 1987; Kang et al., 1987; Robakis et al., 1987; Tanzi et al., 1987). The primary transcript of APP is alternatively spliced to produce several isoforms that encode proteins of different lengths. The APP-695 isoform is highly expressed in the brain, including neurons and glia, while the APP-751 and APP-770 isoforms contain a Kunitz protease inhibitor (KPI) domain and are highly expressed in the periphery (Selkoe, 1998).

Upon synthesis as a holo-protein, APP is proteolytically processed by one of two primary pathways. In the first pathway, APP is cleaved by α-secretase, namely the disintegrin and metalloprotease (ADAM) 10 and 17 proteases, releasing a soluble, extracellular N-terminal fragment (*APP*α) (Asai et al., 2003). The remaining membrane bound C-terminal fragment (C83 or CTFα) is subsequently cleaved by γ-secretase, a protease consisting of of four membrane-
associated subunits, including PSEN1 (or PSEN2), NCSTN, APH-1, and PEN-2, that generates a small peptide, p3, that is subsequently degraded (Fraering et al., 2004). Alternatively, holo-APP can be cleaved by β-secretase or BACE1, a membrane bound aspartyl protease, that releases a slightly smaller N-terminal fragment (APPsβ) (Vassar et al., 1999; Yan et al., 1999). The membrane bound C-terminal fragment (C99 or CTFβ) of APP is subsequently cleaved by γ-secretase, resulting in a small membrane-bound fragment, termed the APP intracellular domain (AICD) as well as the 40 or 42 amino acid Aβ peptides, generated via staggered cleavage by γ-secretase (Figure 1-2).

**Aβ Aggregation**

In 1984, Aβ was identified as the primary constituent of cerebrovascular amyloid found in the brains of individuals with AD as well as DS (Glenner and Wong, 1984b). The next year, Aβ was also identified as the primary component of senile plaques isolated from AD and DS brains (Masters et al., 1985). The amyloid fibrils characteristic of AD are primarily either 40 or 42 amino acids in length, due to staggered cleavage of CTFβ by γ-secretase. Aβ fibrils are observed in a β-pleated sheet structure due to hydrophobic residues at positions 15–21 and 24–32 and a hairpin at positions 22–23 of Aβ. Compared with Aβ40, the Aβ42 species has a higher propensity for aggregation because of two additional hydrophobic residues at the C-terminus (Ile41 and Ala42) of Aβ42 (Jarrett et al., 1993). The aggregation of Aβ and its assembly into fibrils does not occur in a linear biochemical pathway, but rather distinct aggregation
intermediates including Aβ oligomers are formed, some of which appear to subsequently form fibrils (Wetzel, 2006). Initial cleavage of APP by β and γ-secretase results in the production of an Aβ species with a random-coil conformation, but a conformational transition to a β-structure results in Aβ aggregation (Barrow et al., 1992; Chimon et al., 2007; Kirkitadze et al., 2001; Simmons et al., 1994). Once properly folded, Aβ monomers can go on to form oligomers (Aβo) (Chen and Glabe, 2006; Jarrett and Lansbury, 1993). The transition from oligomers to insoluble fibril requires tight packing of the β-pleated sheets, through hydrogen bonding and side-chain interactions (Chimon et al., 2007). An intermediate protofibril stage consists of short, β-sheet rich, rod-like structures that exist in equilibrium with oligomers (Chimon et al., 2007; Walsh et al., 1999). An alternative hypothesis suggests that Aβ oligomers and fibrils are formed by independent pathways (Chen and Glabe, 2006; Necula et al., 2007a; Necula et al., 2007b).

Aβ Clearance

Several pathways that remove Aβ from the brain have been identified, including enzymatic degradation of Aβ, receptor-mediated transport of Aβ out of the brain and phagocytic removal of Aβ (Tanzi et al., 2004).

Several enzymes can degrade Aβ, including two well-studied metalloproteases: the extracellular insulin-degrading enzyme (IDE) and the intracellular membrane metalloendopeptidase (MME) (de Strooper, 2010). Several lines of evidence support the role of IDE in AD pathogenesis. First,
insulin-resistant type II diabetes is associated with an increased risk for AD (Arvanitakis et al., 2004). Second, several studies have demonstrated reduced expression of IDE in human AD brains (Cook et al., 2003; Perez et al., 2000). Third, in transgenic mouse models of AD, loss of one copy of IDE leads to enhanced Aβ deposition while transgenic overexpression leads to reduced Aβ deposition (Farris et al., 2004; Leissring et al., 2003).

MME has also been implicated as playing a role in AD pathogenesis. MME levels decrease with age and loss of function alleles for MME enhance Aβ deposition in transgenic mouse models of AD, while transgenic overexpression of MME results in reduced amyloid plaque burden (Hellstrom-Lindahl et al., 2008; Iwata et al., 2001; Marr et al., 2003). Finally, multiple other enzymes have been implicated in the proteolytic degradation of Aβ although less well studied. This includes endothelin-converting enzymes (ECE) -1 and -2 (Eckman et al., 2001; Eckman et al., 2003; Eckman and Eckman, 2005; Eckman et al., 2006), angiotensin-converting enzyme (ACE) (Hu et al., 2001; Zou et al., 2007), matrix metalloproteases (MMPs) (Yan et al., 2006), plasmin (PA) (Ledesma et al., 2000), and cathepsin B and C (CatB and CatC) (Sun et al., 2008).

Current evidence supports two pathways via which Aβ can move in and out of the brain through the blood brain barrier (BBB). First, several studies support a role for the receptor for advanced glycation end products (RAGE) as facilitating movement of Aβ from the periphery across the BBB into the brain (Lue et al., 2001). Notably, RAGE expression is enhanced in response to Aβ production, leading to enhanced Aβ influx potentially leading a positive feedback
loop in disease progression (Deane et al., 2003; Yan et al., 1996). Second, additional evidence suggests that the low-density lipoprotein receptor-related protein (LRP) binds Aβ and facilitates clearance from the brain to the blood across the BBB (Biere et al., 1996; Shibata et al., 2000). In humans, specific LRP alleles have been associated with increased AD risk (Kang et al., 1997). Furthermore, LRP is present within senile plaques, and LRP expression is reduced in the brain microvessels of AD patients (Donahue et al., 2006; Rebeck et al., 1995). Finally, both *in vitro* and *in vivo* studies have confirmed that LRP-dependent mechanisms clear Aβ from the brain (Deane et al., 2004a; Nazer et al., 2008; Shibata et al., 2000).

Experimental evidence supports the role of phagocytic removal of brain Aβ. First, studies have shown that microglia have the ability to phagocytose Aβ (D’Andrea et al., 2004; Frautschy et al., 1998; Koenigsknecht and Landreth, 2004; Paresce et al., 1996). Furthermore, activated microglia have been shown to degrade Aβ (Majumdar et al., 2007). Second, astrocytes have also been shown to facilitate clearance of Aβ. Aβ has been found in astrocytes in the brains of AD patients (Funato et al., 1998; Nagele et al., 2003). *In vitro* analysis reveals the ability of astrocytes to phagocytose and degrade Aβ (Mandrekar et al., 2009; Koistinaho et al., 2004; Wyss-Coray et al., 2003). Finally, there is evidence that bone marrow-derived monocytes play a role in Aβ clearance. The blood brain barrier (BBB) has been shown to be compromised in mouse models of AD as well as AD patients (Kalaria 1997; Skoog et al., 1998; Wisniewski et al., 1997). Experimental evidence suggests that infiltrating monocytes are capable
of invading the CNS, acquiring microglial morphology and consuming Aβ (Bolmont et al., 2008; Malm et al., 2005; Simard et al., 2006).

**Amyloid Hypothesis**

Aβ is generated during normal cellular processing events but is normally cleared prior to the generation of either Aβ oligomers and fibrils. However, increasing evidence suggests that relatively subtle alterations in either the production or clearance of Aβ dramatically influences AD phenotypes and has led to the amyloid hypothesis of AD that stipulates that it is the absolute amount of Aβ (Aβo) that influences AD risk and is supported by multiple lines of evidence from human AD and mouse models of the disease. First, increased production of full length APP either through trisomy 21 or APP micro-duplications invariably results in AD (Prasher et al., 1998; Rovelet-Lecrux et al., 2006). Second, missense mutations in APP, PSEN1, and PSEN2 result in either overall elevated Aβ levels, preferential production of the more fibrillogenic Aβ42 species, or an increased propensity for fibril formation and all lead to AD in humans (Bateman et al., 2011). For example, one of the most well-studied EOFAD mutations in APP at the β-secretase cleavage site (termed the Swedish mutation due to its identification in a large Swedish pedigree) enhances cleavage by BACE1 and thus increases the levels of all Aβ species (Citron et al., 1992). Notably, transgenic mice containing this EOFAD mutation generate high levels of Aβ and develop age-related Aβ deposits (Hsiao et al., 1996; Lamb et al., 1997). By contrast, multiple EOFAD missense mutations at the C-terminus appear to
modulate the location of γ-secretase cleavage of APP and many of these mutations result in enhanced production of the more fibrillogenic Aβ42 species relative to the production of the Aβ40 species (Maruyama et al., 1996). Transgenic mice containing these mutations develop age-related deposits with dramatically enhanced Aβ42 levels (Games et al., 1995; Moechars et al, 1999). Mutations within the Aβ sequence itself seem to alter the conformation of the peptide and its propensity to form Aβ aggregates (Bateman et al., 2011; Nilsberth et al., 2001). Notably, immunohistochemical studies have revealed an increased abundance of cerebrovascular amyloid with many of these mutations (Nilsberth et al., 2001). Transgenic mouse models with these mutations develop age-related Aβ deposits and, in many cases, cerebral amyloid angiopathy (CAA) (Knobloch et al., 2007; Ronnback et al., 2011). Finally, many of the mutations in presenilin alter the ratios of Aβ42 to Aβ40 due to either elevated production of Aβ42 or decreased production of Aβ40, thus facilitating Aβ aggregation and deposition in these families (Bateman et al., 2011; Kumar-Singh et al., 2006).

Finally, there is increasing evidence that Aβ peptides can accumulate due to decreased Aβ clearance (Tanzi et al., 2004). Analysis of patients with AD reveals down-regulation of enzymes that degrade Aβ (IDE, MME) as well as receptors that move Aβ out of the brain (LRP) (Cook et al., 2003; Donahue et al., 2006; Russo et al., 2005). Furthermore, GWASs have shown altered expression in several genes that may be involved in Aβ clearance, including BIN1 (bridging integrator 1), CLU (clusterin), CR1 (complement component [3b/4b] receptor 1), and PICALM (phosphatidylinositol binding clathrin assembly protein).
(Carrasquillo et al., 2010; Harold et al., 2009; Lambert et al., 2009; Schjeide et al., 2009; Seshadri et al., 2010). Studies have also shown that ε4 allele of APOE, a genetic risk factor for AD, is less efficient at clearance of brain Aβ (Deane et al., 2008; Jiang et al., 2008).

Based on genetic findings as well as experimental evidence regarding production and accumulation of Aβ and its association with AD in humans, the amyloid cascade hypothesis was proposed (Selkoe 1996; Hardy and Selkoe, 2002). The main premise of the hypothesis is that chronic imbalance between Aβ production and clearance leads to Aβ aggregation in the form of Aβo, protofibrils, or fibrils, which initiates a complex cascade of events, including inflammatory response, oxidative injury, tau aggregation, and neuronal/synaptic dysfunction, which culminates in memory loss (Figure 1-3) (Selkoe, 2005b; Selkoe, 2008).

Mouse Models of Alzheimer’s Disease

Although human studies reveal a high degree of heritability of AD risk, many of the genes responsible for AD remain to be identified. Candidate gene and GWASs have yet to identify a majority of the genetic risk factors associated with AD. While meta-analysis has increased the power of individual case-control studies, their success at reliably identifying genetic risk factors for AD remains to be determined. As a result, many laboratories have turned to model organisms, the mouse in particular, to identify genetic factors that influence the development and progression of AD phenotypes. These mouse models offer several
advantages, including; short generation time, the ability to control for genetic backgrounds and environment, the ease of obtaining large population size for increased power in identifying genes responsible for small effects, and ease of assaying brain samples. In order to test various aspects of the amyloid hypothesis, numerous groups have generated transgenic mouse models of AD with EOFAD mutations.

The discovery of autosomal dominant mutations that cause AD as well as the advent of technologies to alter gene expression in the mouse through exogenous addition of mutant human genes into the mouse genome (transgenesis) or modification of the endogenous mouse genome (gene targeting) created the opportunity to model AD in the mouse. The first report of a transgenic mouse model with robust $A\beta$ pathology was the PDAPP mouse (Games et al., 1995). This model contains a human $APP$ mini-gene with the London EOFAD mutation (V717F), driven by the platelet-derived growth factor-$\beta$ (PDGF) promoter. The PDAPP mouse displays amyloid deposition beginning at 6 to 9 months of age and develops extensive dystrophic neurites, reactive astrocytes, activated microglia, age-related learning deficits, and synaptic loss (Chen et al., 2000; Dodart et al., 2000; Reilly et al., 2003). The following year, the Tg2576 mouse, the most widely studied mouse model of AD, was described by Karen Ashe and colleagues (Hsiao et al., 1996). Tg2576 uses the hamster prion promoter (Prp) to drive expression of a human $APP$ transgene containing the Swedish EOFAD familial mutation (K670N/M671L) in the central nervous system (Hsiao et al., 1996). These animals develop age-dependent amyloid
plaque pathology that correlates with memory deficits. Many other transgenic lines have been developed by similar approaches, expressing \(APP\) transgenes and/or \(PSEN1\) transgenes with one or more familial mutations (Table 1-3). Notably, that same year, a genomic-based transgenic model was generated using gene targeting to introduce the Swedish EOFAD mutation into the CD-1 outbred strain (Reaume et al., 1996). The following year, another genomic-based transgenic model (R1.40) was characterized (described below). While models containing mutant human \(PSEN1\) and \(PSEN2\) transgenes do not develop A\(\beta\) pathology on their own, combining the mutant \(PSEN1\) transgenes with various mutant \(APP\) transgenes resulted in earlier and more extensive plaque formation (Borchelt et al., 1996; Borchelt et al., 1997; Holcomb et al., 1998). Finally, while not covered in this thesis, it is important to note that transgenic mouse models of AD containing human tau in combination with other transgenes were subsequently generated (Oddo et al., 2003). The ease of generating transgenic models of human disease has greatly facilitated the testing of specific hypothesis regarding AD, including the amyloid hypothesis. Two of these mouse models of AD, the R1.40 congenic line and the APPPS1 line, are the focus of the current thesis.

**R1.40**

The R1.40 mouse model of AD contains a genomic-based transgene with the entire human \(APP\) promoter, transcriptional regulatory elements, and appropriate splice donor and acceptor sites. The \(APP\) YAC R1.40 transgene
contains the Swedish EOFAD double mutation, K670N/M671L. The animal has six to eight copies of the APP transgene inserted into mouse chromosome 13 (Kulnane et al., 2002). These mice generate the entire spectrum of alternatively spliced transcript and protein isoforms of APP in a temporal and spatial pattern that resembles that observed in the human brain. Homozygous R1.40 animals express human-derived transgene products at four- to six-fold the level of endogenous mouse App (Lamb et al., 1999). This model displays many of the pathological hallmarks of AD, including: Aβ deposits, dystrophic neurites, hyperphosphorylated tau, and inflammation (Kulnane and Lamb, 2001). Aged R1.40 animals also exhibit behavioral impairments on spontaneous alteration, water maze retention, and interference on the retention of an odor-guided task (Hock et al., 2009). In an effort to examine the influence of genetic background on AD phenotypes, the APP YAC R1.40 transgene was backcrossed onto four different mouse genetic backgrounds (C57BL/6J, DBA2/J, A/J, and 129S1/Sv1mJ) for more than 10 generations, generating congenic R.140 lines (B6-R1.40, D2-R1.40, A-R1.40, and 129S1-R1.40, respectively) with identically inserted transgenes. Studies have revealed that B6-R1.40 animals exhibit high levels of steady state brain Aβ early in life, leading to Aβ deposition later in life (starting at 13 months). By contrast, it was previously reported that D2-R1.40 animals have reduced steady state Aβ levels compared with B6-R1.40 animals, with no detectable Aβ deposition at 13 months of age (Lehman et al., 2003a). Based on the differences between the steady state Aβ levels between the B6-R1.40 and D2-R1.40 mice, a F2 genetic mapping study was performed to identify
genetic loci associated with altered levels of brain Aβ. The results of these studies identified a highly significant association with a locus on mouse chromosome 2 as well as additional genetic associations on mouse chromosomes 1 and 7 (Ryman et al., 2008).

**APPSS1**

The APPSS1 mouse model of AD was generated by co-injection of two cDNA constructs (Thy1-APPKM670/671NL and Thy1-PS1L166P) into C57BL/6J embryos. The *APP* and *PSEN1* transgenes in this model are driven by the Thy1 promoter, which is expressed in postnatal neurons (Radde et al., 2006). The APPSS1 model has many of the pathological hallmarks associated with AD, including Aβ deposits, dystrophic neurites, hyperphosphorylated tau, microglial activation, and synaptic loss. This model begins to exhibit Aβ deposits at 2 months of age, providing a useful tool for a genetic screen of Aβ deposition as described in this thesis.

**Effects of Diet on APP/Aβ in Mouse Models of AD**

There have been several studies in transgenic mouse models of AD examining the effects of a hypercholesterolemic diet on serum cholesterol levels, APP processing, Aβ metabolism and deposition (Figure 1-4) (Fitz et al., 2010; George et al., 2004; Hooijmans et al., 2007; Howland et al., 1998; Levin-Allerhand et al., 2002; Li et al., 2003; Pedrini et al., 2009; Refolo et al., 2000; Shie et al., 2002; Shie et al., 2003). Not surprisingly, all showed an increase in
However, the effects of high-fat/high-cholesterol diets (HF/HC) on the levels of brain Aβ in mouse models of AD have provided conflicting results. Several studies have shown that HF/HC diet significantly increases brain Aβ levels, based on either Aβ ELISA or Aβ deposition (Fitz et al., 2010; Hooijmans et al., 2007; Levin-Allerhand et al., 2002; Li et al., 2003; Refolo et al., 2000; Shie et al., 2002; Shie et al., 2003). However, other studies have shown that a HF/HC diet can actually reduce the levels of brain Aβ (George et al., 2004; Howland et al., 1998; Van der Auwera et al., 2005).

While only a subset of studies have analyzed the effect of HF/HC diet on other parameters of AD such as APP processing, APOE levels and behavior, they too have had mixed results. One study demonstrated that a HF/HC diet resulted in an elevation of serum cholesterol, brain Aβ, and APP CTFβ, as well as a reduction in APPsα, suggesting increased β-secretase processing of APP may be responsible for changes in Aβ observed on the HF/HC diet (Refolo et al., 2000). However, other studies suggested that, while Aβ levels were increased, the HF/HC diets did not alter APP processing as demonstrated by unaltered levels of APPsα and no detectable alterations in cell-associated APP or APP CTFs (Fitz, 2010; Levin-Allerhand et al., 2002). Furthermore, when looking at the effects of hypercholesterolemia in mouse models of AD, three studies
demonstrated that the levels of brain APOE directly correlated with the levels of Aβ, while one study showed an inverse correlation (Fitz et al., 2010; Howland et al., 1998; Levin-Allerhand et al., 2002; Shie et al., 2002). Finally, some studies have attempted to correlate the effect of HF/HC diet on behavior. These studies revealed increased serum cholesterol and increased brain Aβ resulted in behavioral deficits (Fitz et al., 2010; Li et al., 2003).

Lowering cholesterol levels in mouse models of AD appears to support cholesterol as a risk factor for AD. Treatment of HF/HC fed APP transgenic animals with a liver X receptor (LXR) antagonist ameliorates the effects of a HF/HC diet, reducing Aβ deposition and improving cognition (Fitz et al., 2010). Likewise, restriction of cholesterol along with oleic acid supplementation in APP transgenic animals reduced the levels of Aβ deposits perhaps due to changes in the steady state amounts of Aβ40 and Aβ42 (Amtul et al., 2011). However, the use of statins in mouse models of AD has yielded conflicting findings. One study demonstrated that statin treatment of a mouse model of AD reduced total cholesterol, reduced Aβ and reduced Aβ deposition (Petanceska et al., 2002). However, a second study showed that statin treatment resulted in reduced cholesterol levels, but had no effect on Aβ levels in males and actually increased Aβ levels and Aβ deposition in females (Park et al., 2003). A third study showed that treatment of a transgenic mouse model with statins reduced serum cholesterol and improved cognition, but had no effect Aβ levels (Li et al., 2006). Finally, another study showed that while statins alone had no effect, treatment of APP transgenic animals with statin and D-4F, an Apo A-I mimetic peptide,
reduced amyloid plaques and improved cognition (Handattu et al., 2009).

In summary, similar to the conflicting epidemiological studies in humans, the studies examining the link between cholesterol and AD phenotypes in the mouse have yielded inconsistent findings. It remains unclear whether the different outcomes obtained in these studies is due to alterations in the diets, promoters, transgenes, familial AD mutations, genetic background or drug utilized in these studies.

**Research Aims**

Even though AD was first described over 100 years ago and there is a broad body of research providing links to specific disease mechanisms, many questions remain. Although the genetics of early onset AD (EOFAD) clearly links APP and Aβ to the underlying disease mechanisms, the exact role of APP and Aβ in AD remains unknown. While a large number of human genetic and epidemiological studies have attempted to exhaustively identify the genetic and environmental factors that influence AD risk, it remains clear that a majority of the factors influencing the most common form of AD (LOFAD) remain to be identified. This thesis aimed to examine the role of genes and environment in AD phenotypes, focusing on the levels of brain Aβ, through the use of well-defined mouse models of AD. Ultimately, a better understanding of the genetic and environmental risk factors for AD should lead to better treatments for the disease.

Previous studies have demonstrated that genetic background can alter AD risk. Likewise, a hypercholesterolemic diet is a postulated environmental risk
factor for AD. In this thesis, I first examined whether a postulated risk factor for AD (HF/HC diets) influenced AD phenotypes that was dependent upon the genetic background of the animals (Chapter 2). In particular, I examined the effect HF/HC diets, on APP processing and steady state Aβ levels early in life in three of the congenic R1.40 lines (B6-R1.40, D2-R1.40, and 129S1-R1.40). It was discovered that diet influenced Aβ levels only in the B6-R1.40 strain, but not the other three strains containing the identical APP transgene. Furthermore, a unique gene expression profile was identified in the brains of B6-R1.40 mice on the HF/HC diets, that was not observed in the other strains. This study suggests that genetic background dramatically alters the environmental influence of high-fat/high-cholesterol diets on Aβ metabolism within the brain, which could have implications for similar dietary factors influencing human AD risk.

The second set of experiments, expands on the previous QTL mapping study between the B6-R1.40 and D2-R1.40 lines that identified a region of mouse chromosome 2 containing loci involved in reduced Aβ metabolism (Chapter 3). A B6-R1.40 animal containing a DBA/2J congenic region correlating to the associated QTL region was analyzed for Aβ metabolism and Aβ deposition. This study provided evidence for the role of the Complement C5 gene in modifying Aβ deposition.

The third set of experiments expands on previous studies examining the effect of genetic background on Aβ metabolism in the R1.40 mouse model of AD and narrows in on the genetic loci that alter Aβ metabolism and deposition in the mouse model of AD using several different strategies (Chapter 4). The initial
studies examining genetic factors that influence Aβ deposition focused on B6-R1.40 and D2-R1.40 animals. The current studies examine all of the congenic R1.40 lines (B6-R1.40, D2-R1.40, 129S1-R1.40, and A-R1.40), characterizing the insertion site and examining APP processing, Aβ metabolism, and Aβ deposition throughout life. Our results suggest the presence of genetic modifiers on the 129S1, DBA/2J, and A/J genetic background that delay and, in some cases, prevent Aβ deposition. This thesis demonstrates identical insertion of the R1.40 transgene on four different inbred strains can result in dramatically altered disease phenotypes. Consomic strains, in which a single A/J chromosome was substituted into an otherwise C57BL/6J background, were used to identify genes on the A/J background that reduce Aβ deposition. These studies focused on the analysis of three chromosomes that had previously been implicated in regulating Aβ deposition. Congenic strains, in which specific segments of the DBA/2J strain were introduced into an otherwise C57BL/6J background, were used to identify genes responsible for altered Aβ metabolism and deposition in the D2-R1.40 strain. The congenic strains utilized, corresponded to the remaining chromosomal regions (mouse chromosome 1 and 7) implicated in the previous genetic mapping study.

In summary, human genetic studies of AD have proven limited in their ability to clearly identify genes involved in LOFAD. This thesis demonstrates that unique genetic loci inherited in common inbred strains of mice have multiple influences on AD phenotypes, from alterations in Aβ metabolism to deposition of Aβ as well as impacting the response to environmental factors such as a high-
fat/high-cholesterol diets. In addition, these studies provide evidence for one particular genetic modifier, namely C5, in regulating Aβ deposition in the mouse and provide evidence that the mouse is an effective method to identify genetic factors associated with altered Aβ metabolism in LOFAD.
Alzheimer's Disease

Environmental
(\sim 20\%)
Cholesterol
TBI
Education
Exercise

Genetic
(\sim 80\%)

Early Onset
(Familial)
(1\%)

Late Onset

Autosomal Dominant
Mutation
APP (Ch21)
PSEN1 (Ch14)
PSEN2 (Ch1)

Dosage Imbalance
Trisomy 21
APP duplication

APOE
(20-30\%)
CLU
PICALM
CR1
SORL1
?
Figure 1-1: Genetic and environmental factors influence AD risk. AD risk is estimated to be comprised of genetic (~80%) and environmental (~20%) factors. The genetics of early onset AD (EOFAD) is well established, but accounts for <1% of all cases of AD. The majority of cases of AD are late onset AD (LOFAD). There have been links to several chromosomes, but the only gene definitively associated with LOFAD is APOE. It has been estimated that over 70% of the genetic risk factors for AD have yet to be discovered (Gatz et al., 2006).
Table 1-1: Genes showing genome-wide significance in at least one GWAS.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Chromosome</th>
<th>Initial GWAS Reference</th>
<th>GWAS p-value</th>
<th>GWAS OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATXN1</td>
<td>Ataxin 1</td>
<td>6p23</td>
<td>(Bertram et al., 2008)</td>
<td>5x10^-3</td>
<td>not listed</td>
</tr>
<tr>
<td>BIN1*</td>
<td>Bridging integrator 1</td>
<td>2q14</td>
<td>(Seshadri et al., 2010)</td>
<td>3.0x10^-10</td>
<td>1.5</td>
</tr>
<tr>
<td>CD33</td>
<td>CD33 antigen</td>
<td>19q13.3-q13.4</td>
<td>(Bertram et al., 2008)</td>
<td>4.88x10^-6</td>
<td>not listed</td>
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<tr>
<td>CLU*</td>
<td>Clusterin</td>
<td>8p21-p12</td>
<td>(Harold et al., 2009)</td>
<td>1.4x10^-9</td>
<td>0.84</td>
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<td>CR1*</td>
<td>Complement component receptor 1</td>
<td>1q32</td>
<td>(Harold et al., 2009)</td>
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<td>EXOC3L2</td>
<td>Exocyst complex component 3-like 2</td>
<td>19q13.32</td>
<td>(Seshadri et al., 2010)</td>
<td>2.1x10^-10</td>
<td>1.5</td>
</tr>
<tr>
<td>GAB2</td>
<td>GRB2-associated binding protein 2</td>
<td>11q13.4-q13.5</td>
<td>(Reiman et al., 2007)</td>
<td>9x10^-11</td>
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<tr>
<td>MTHFD1L</td>
<td>Methylene tetrahydrofolate dehydrogenase, NADP(+)-dependant 1-like</td>
<td>6q25.2</td>
<td>(Naj et al., 2010)</td>
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<td>PCDH11X</td>
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<td>Phosphatidylinositol-binding clathrin assembly protein</td>
<td>11q14</td>
<td>(Harold et al., 2009)</td>
<td>1.9x10^-8</td>
<td>0.85</td>
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</table>

* confirmed by extensive and independent replication data (Carrasquillo et al., 2010; Jun et al., 2010; Lambert et al., 2009; Schjeide, 2009)
Table 1-2: Current top nine genes, in addition to APOE, associated with Alzheimer’s disease based on meta-analyses of published association studies (alzgene.org, October 2010).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Chromosome</th>
<th># of Studies</th>
<th>OR* (95% CI)</th>
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<tbody>
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<td>CLU</td>
<td>Clusterin</td>
<td>8p21-p12</td>
<td>13</td>
<td>0.88 (0.86, 0.91)</td>
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<tr>
<td>PICALM</td>
<td>Phosphatidylinositol-binding clathrin assembly protein</td>
<td>11q14</td>
<td>7</td>
<td>0.88 (0.85, 0.91)</td>
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<td>EXOC3L2</td>
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<td>1.17 (1.12, 1.23)</td>
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<td>4</td>
<td>1.15 (1.10, 1.20)</td>
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<tr>
<td>CR1</td>
<td>Complement component receptor 1</td>
<td>1q32</td>
<td>11</td>
<td>1.14 (1.08, 1.20)</td>
</tr>
<tr>
<td>SORL1</td>
<td>Sortilin-related receptor</td>
<td>11q23.2-q24.2</td>
<td>21</td>
<td>1.10 (1.03, 1.17)</td>
</tr>
<tr>
<td>GWA_14q32.13</td>
<td>rs11622883</td>
<td>14q32.13</td>
<td>5</td>
<td>0.84 (0.77, 0.93)</td>
</tr>
<tr>
<td>TNK1</td>
<td>Tyrosine kinase, nonreceptor, 1</td>
<td>17p13.1</td>
<td>5</td>
<td>0.84 (0.76, 0.93)</td>
</tr>
<tr>
<td>IL8</td>
<td>Interleukin 8</td>
<td>4q12-q13</td>
<td>4</td>
<td>1.27 (1.08, 1.50)</td>
</tr>
</tbody>
</table>

*Odds ratio (OR) is a measure of the gene’s effect size. The average effect size for APOE ~ 3-4, for risk alleles ~1.25, and for protective alleles ~0.8 (Bertram and Tanzi, 2008).
A. **NON-AMYLOIDGENIC**

\[ \text{NH}_2 \xrightarrow{\alpha\text{-secretase}} \text{CTF-}\alpha \xrightarrow{\gamma\text{-secretase}} \text{COOH} \]

\[ \text{APPs}_\alpha \xrightarrow{\beta\text{-secretase}} \text{p3} \xrightarrow{\text{cleared}} \]

B. **AMYLOIDGENIC**

\[ \text{NH}_2 \xrightarrow{\beta\text{-secretase}} \text{CTF-}\beta \xrightarrow{\gamma\text{-secretase}} \text{COOH} \]

\[ \text{APPs}_\beta \xrightarrow{\text{aggregated}} \text{A}\beta \]
Figure 1-2: Processing of the amyloid precursor protein (APP). A) Primary cleavage by \( \alpha \)-secretase followed by secondary cleavage by \( \gamma \)-secretase results in cleavage products APP\(_{\alpha} \), CTF\(_{\alpha} \), and amino acid peptide, p3. B) Primary cleavage by \( \beta \)-secretase followed by secondary cleavage by \( \gamma \)-secretase results in cleavage products APP\(_{\beta} \), CTF\(_{\beta} \), and 40 or 42 amino acid peptides, A\( \beta \)40/42 (Selkoe, 1999).
Down Syndrome

APP locus duplication

APP or PSEN mutations

ApoE
RAGE
LRP
MME
IDE

Aβ40

Aβ42

Oligomers

Plaques

Inflammation

Oxidative Stress

Neurofibrillary Tangles

Neuronal/Synaptic Dysfunction

Dementia
Figure 1-3: The amyloid hypothesis. According to the amyloid hypothesis, accumulation of Aβ in the brain is the primary influence driving AD pathogenesis and the rest of the disease process is the result of an imbalance between Aβ production and Aβ clearance. Evidence to support this theory includes the unique pathological hallmark of AD, Aβ deposits (senile plaque), and genetic evidence that alterations in APP are disease causative. Increased dosage of APP due to trisomy or duplications causes AD. Likewise, mutations in the APP gene and as well as genes encoding proteins (PSEN1, PSEN2) involved in its proteolytic processing are known to cause disease. There is also evidence that alterations in Aβ turnover (APOE, RAGE, LRP, IDE, MME) influences disease. The Aβ peptides aggregate to form oligomers and fibrils, initiating a cascade of events, including inflammatory changes, oxidative injury, altered kinase/phosphatase activity resulting in tangles, neuritic/synaptic changes, neuronal loss and altered cognition [adapted from (Selkoe, 2005a)].
### Table 1-3: Selected examples of mouse models of Alzheimer’s disease.

<table>
<thead>
<tr>
<th>Line</th>
<th>Promoter</th>
<th>FAD Mutation</th>
<th>Genetic Background</th>
<th>Amyloid Pathology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>APPSWE (Tg2576)</td>
<td>PrP</td>
<td>APPK670N/M671L</td>
<td>F2: C57BL/6, SJL</td>
<td>9-10 months</td>
<td>(Hsiao et al., 1996)</td>
</tr>
<tr>
<td>PDAPP</td>
<td>PDGF</td>
<td>APPV717F</td>
<td>C57BL/6, DBA, Swiss Webster</td>
<td>6-9 months</td>
<td>(Games et al., 1995)</td>
</tr>
<tr>
<td>APP23</td>
<td>Thy-1</td>
<td>APPK670N/M671L</td>
<td>C57BL/6, DBA/2</td>
<td>6 months</td>
<td>(Calhoun et al., 1999)</td>
</tr>
<tr>
<td>TgCRND8</td>
<td>PrP</td>
<td>APPK670N/M671L APPV717F</td>
<td>C57BL/6, C3H</td>
<td>3 months</td>
<td>(Chishti et al., 2001)</td>
</tr>
<tr>
<td>PSAPP</td>
<td>PDGF</td>
<td>APPK670N/M671L PS1M146V</td>
<td>C57BL/6, SJL, DBA, Swiss Webster</td>
<td>2.5 months</td>
<td>(McGowan et al., 1999)</td>
</tr>
<tr>
<td>TgAPPSwe-KI</td>
<td>mApp</td>
<td>APPK670N/M671L</td>
<td>CD-1</td>
<td>none</td>
<td>(Reaume et al., 1996)</td>
</tr>
<tr>
<td>APP London</td>
<td>Thy-1</td>
<td>APPV717I</td>
<td>FVB</td>
<td>10-12 months</td>
<td>(Moechars et al., 1999)</td>
</tr>
<tr>
<td>APPPS1</td>
<td>Thy-1</td>
<td>APPK670N/M671L PS1L166P</td>
<td>C57BL/6J</td>
<td>2 months</td>
<td>(Radde et al., 2006)</td>
</tr>
<tr>
<td>B6-R1.40</td>
<td>Human Genomic</td>
<td>APPK670N/M671L</td>
<td>C57BL/6J</td>
<td>13 months</td>
<td>(Lamb et al., 1997)</td>
</tr>
<tr>
<td>A-R1.40</td>
<td>Human Genomic</td>
<td>APPK670N/M671L</td>
<td>A/J</td>
<td>Never</td>
<td>Unpublished</td>
</tr>
<tr>
<td>129S1-R1.40</td>
<td>Human Genomic</td>
<td>APPK670N/M671L</td>
<td>129S1/SvlmJ</td>
<td>24 months</td>
<td>Unpublished</td>
</tr>
<tr>
<td>D2-R1.40</td>
<td>Human Genomic</td>
<td>APPK670N/M671L</td>
<td>DBA/2J</td>
<td>24 months</td>
<td>Unpublished</td>
</tr>
</tbody>
</table>
Table 1-4: Summary of studies examining the effects of HF/HC diet on mouse models of AD.

<table>
<thead>
<tr>
<th>Transgenic Model</th>
<th>Genetic Background</th>
<th>Promoter</th>
<th>Mutation</th>
<th>Gender</th>
<th>Fat (kcal)</th>
<th>Chol. (g)</th>
<th>Cholate (g)</th>
<th>Start</th>
<th>Duration</th>
<th>Body Weight</th>
<th>Chol.</th>
<th>Abeta</th>
<th>Plaque</th>
<th>APP Processing</th>
<th>memory</th>
<th>CAA</th>
<th>APOE</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1.40</td>
<td>C57BL/6</td>
<td>endog</td>
<td>swedish</td>
<td>m/f</td>
<td>40%</td>
<td>1.25%</td>
<td>-</td>
<td>4 wk</td>
<td>8 wk</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>-</td>
<td>NC</td>
<td>-</td>
<td>-</td>
<td>NC</td>
<td>unpublished</td>
</tr>
<tr>
<td>R1.40</td>
<td>DBA/2J</td>
<td>endog</td>
<td>swedish</td>
<td>m/f</td>
<td>40%</td>
<td>1.25%</td>
<td>-</td>
<td>4 wk</td>
<td>8 wk</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>NC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>unpublished</td>
</tr>
<tr>
<td>R1.40</td>
<td>129</td>
<td>endog</td>
<td>swedish</td>
<td>m/f</td>
<td>40%</td>
<td>1.25%</td>
<td>-</td>
<td>4 wk</td>
<td>8 wk</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>NC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>unpublished</td>
</tr>
<tr>
<td>R1.40</td>
<td>A/J</td>
<td>endog</td>
<td>swedish</td>
<td>m/f</td>
<td>40%</td>
<td>1.25%</td>
<td>-</td>
<td>4 wk</td>
<td>8 wk</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>NC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>unpublished</td>
</tr>
<tr>
<td>Tg2576</td>
<td>90% SJL</td>
<td>prion</td>
<td>swedish</td>
<td>f</td>
<td>37%</td>
<td>1.25%</td>
<td>5%</td>
<td>2 mo</td>
<td>7-10 mo</td>
<td>-</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↑</td>
<td>Shi et al., 2002, Shi et al., 2003</td>
</tr>
<tr>
<td>Tg2576</td>
<td>C57BL/6</td>
<td>prion</td>
<td>swedish</td>
<td>m/f</td>
<td>37%</td>
<td>1.25%</td>
<td>0.5%</td>
<td>7-9 mo</td>
<td>4 mo</td>
<td>-</td>
<td>↑</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↓</td>
<td>↑</td>
<td>-</td>
<td>Li et al., 2003</td>
</tr>
<tr>
<td>Tg2576</td>
<td>F2 B6xSJL</td>
<td>prion</td>
<td>swedish</td>
<td>f</td>
<td>32%</td>
<td>5%</td>
<td>2%</td>
<td>12 mo</td>
<td>6 wk</td>
<td>NC</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>AICD, ↑</td>
<td>sAPPa</td>
<td>-</td>
<td>-</td>
<td>George et al., 2004</td>
</tr>
<tr>
<td>PSAPP (Tg2576xPS1)</td>
<td>B6 SJL/SW</td>
<td>prion/</td>
<td>PDGFβ2</td>
<td>m</td>
<td>32%</td>
<td>5%</td>
<td>2%</td>
<td>5 wk</td>
<td>7 wk</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>bctf, ↑</td>
<td>sAPPa</td>
<td>-</td>
<td>-</td>
<td>Refolo et al., 2000</td>
</tr>
<tr>
<td>gene-targeted</td>
<td></td>
<td>APP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Howland et al., 1998</td>
</tr>
<tr>
<td>APP/Ld</td>
<td>FVB</td>
<td>thy-1</td>
<td>london</td>
<td>f</td>
<td>94%*</td>
<td>-</td>
<td>-</td>
<td>3 mo</td>
<td>43 d</td>
<td>↓</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Van der Auwera et al., 2005</td>
</tr>
<tr>
<td>APPswe (Borchelt)</td>
<td>C57BL/6</td>
<td>prion</td>
<td>swedish</td>
<td>m</td>
<td>42%</td>
<td>0.2%</td>
<td>-</td>
<td>6 wk</td>
<td>10.5 mo</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>-</td>
<td>NC</td>
<td>↑</td>
<td>-</td>
<td>-</td>
<td>Levin-Allerhand et al., 2002</td>
</tr>
<tr>
<td>APPswe/ PS1-DE9</td>
<td>C57BL/6</td>
<td>prion</td>
<td>swedish</td>
<td>ps1-DE9</td>
<td>44%</td>
<td>1%</td>
<td>-</td>
<td>6 mo</td>
<td>12 mo</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>-</td>
<td>-</td>
<td>NC</td>
<td>-</td>
<td>-</td>
<td>Hooijmans et al., 2007</td>
</tr>
<tr>
<td>TgCRND8</td>
<td>C3H/He</td>
<td>prion</td>
<td>swedish</td>
<td>m/f</td>
<td>60%*</td>
<td>-</td>
<td>-</td>
<td>4 wk</td>
<td>18 wk</td>
<td>↑</td>
<td>-</td>
<td>↑</td>
<td>NC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Pedrini et al., 2009</td>
</tr>
<tr>
<td>APP23</td>
<td>C57BL/6</td>
<td>thy-1</td>
<td>swedish</td>
<td>m/f</td>
<td>41%</td>
<td>0.2%</td>
<td>-</td>
<td>8-10 mo</td>
<td>4 mo</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>NC</td>
<td>↓</td>
<td>↑</td>
<td>-</td>
<td>Fitz et al., 2010</td>
</tr>
</tbody>
</table>
Chapter 2: Gene-Environment Interactions Influence Beta-Amyloid Metabolism in Mice

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* Submitted for Publication
ABSTRACT

Increasing evidence suggests that Alzheimer’s disease (AD) is due to a complex interplay between both genetic and environmental influences. Previous studies have provided genetic evidence supporting the role of brain beta-amyloid (Aβ) peptide in AD pathogenesis, as well as implicating high-fat/high-cholesterol (HF/HC) diets as an environmental risk factor for AD. The present study examined the effect of HF/HC diets on the R1.40 mouse model of AD maintained on multiple inbred strains (C57BL/6J, 129S1/SvImJ and DBA/2J). The HF/HC diet increased body weight and serum cholesterol levels in all strains, while steady state brain Aβ levels were increased only in the C57BL/6J animals. Notably, the levels of Aβ were elevated despite unchanged levels of the amyloid precursor protein, total brain cholesterol and apolipoprotein E. Gene expression microarray analysis revealed a unique set of genes differentially regulated in the C57BL/6J genetic background in response to diet. These studies demonstrate that the effect of HF/HC diets is dependent upon the genetic background examined and has implications for future studies of human AD.

INTRODUCTION

Alzheimer’s disease (AD), the most common dementing disorder of late life, is a major cause of disability and death in the elderly. A definitive diagnosis of AD requires the demonstration of neuropathological hallmarks including extracellular deposits of the β-amyloid (Aβ) protein (senile plaques) in a characteristic pattern within the brain parenchyma (1997). Numerous
epidemiological studies have demonstrated that AD is a complex, heterogeneous disorder with numerous postulated genetic and environmental risk factors (Bertram and Tanzi, 2008).

Detailed studies of a large registry of Swedish twins have revealed a substantially higher concordance of AD in monozygotic twins when compared to dizygotic twins. Based upon these studies, it has been estimated that upwards of 40-80% of AD is due to inherited/genetic factors, with the balance explained by ill-defined environmental and stochastic factors (Gatz et al., 1997; Gatz et al., 2006; Raiha et al., 1996). Genetic investigations have identified multiple etiologies for AD and provided strong evidence that the genetic forms of AD share common pathogenic mechanisms that involve relatively subtle alterations in the release of Aβ from its larger precursor, the amyloid precursor protein (APP). These observations have supported the “amyloid cascade hypothesis” of AD, which stipulates that it is the absolute levels of specific Aβ peptides or Aβ oligomers (Aβo) that dictate AD risk, although the exact relationship between Aβ and other disease phenotypes remains quite unclear (Selkoe, 2008).

Retrospective epidemiological studies have provided evidence for a large number of environmental factors that modify risk for developing AD. These include exposure to environmental toxins such as metals and pesticides, head trauma, physical activity, level of educational attainment as well as dietary factors (Burgener et al., 2008; Butler et al., 1996; Jellinger, 2004; Rolland et al., 2008; Santibanez et al., 2007; Shcherbatyk and Carpenter, 2007). The mechanistic relationship between these postulated environmental risk factors and the known
genetic factors have proven exceedingly difficult to study.

Several lines of evidence suggest that one of the environmental risk factors for AD is altered cholesterol metabolism. First, elevated cholesterol levels have been associated with increased susceptibility to AD and with higher senile plaque load in AD subjects in some, but not all, studies (Anstey et al., 2008; Stewart et al., 2007; Whitmer et al., 2005). Not surprisingly, atherosclerosis, in which elevated serum cholesterol is a predisposing factor, has also been shown to correlate with greater AD risk (Hofman et al., 1997; Honig et al., 2005).

Second, allelic variation at the APOE locus represents the most significant genetic risk for late-onset AD (Farrer et al., 1997). APOE is one the major apolipoproteins in serum and is the major cholesterol carrier in the brain (Mahley, 1988). The ε4 APOE allele, which has increased binding affinity for both cholesterol and the LDL receptor, has been strongly associated with increased relative risk for AD (Corder et al., 1993).

Third, retrospective cohort studies of human patients receiving lipid-lowering statin drugs (inhibitors of HMGCoA reductase, a key enzyme in cholesterol biosynthesis) have demonstrated an important side benefit of statins in reducing the risk for Alzheimer’s disease (Haag et al., 2009; Li et al., 2007; Wolozin et al., 2000). It is not known whether this effect is specific to this class of drugs. It is also unknown if the effects in reducing AD results directly from statins’ influence on blood lipid profile, their anti-inflammatory properties or other off target effects. Regardless, human studies support a role of cholesterol-
lowering drugs in reducing the risk of AD (Jick et al., 2000; Wolozin et al., 2007).

Finally, several in vitro and in vivo studies have suggested a link between altered cholesterol and Aβ metabolism [reviewed in (Puglielli et al., 2003)]. In vitro experiments specifically modifying cellular cholesterol levels have demonstrated that Aβ production is positively correlated with membrane cholesterol levels. However, in vivo studies exposing cDNA-based transgenic mouse models of AD to diets high in fat and extremely high in cholesterol as well as to statin therapies have had contradictory results, with studies demonstrating both positive and negative correlations between serum cholesterol levels and Aβ metabolism and deposition (George et al., 2004; Hooijmans et al., 2007; Howland et al., 1998; Levin-Allerhand et al., 2002; Li et al., 2003; Park et al., 2003; Pedrini et al., 2009; Refolo et al., 2000; Shie et al., 2002; Shie et al., 2003; Van der Auwera et al., 2005). These animal studies are confounded by the utilization of mice with various mixed genetic backgrounds and high dietary concentrations of cholic acid, an atherogenic compound with significant neurotoxicity.

The R1.40 mouse model of AD was used to examine whether the effect of defined high-fat/high-cholesterol diets on Aβ metabolism is altered dependent upon genetic background. R1.40 contains a complete genomic copy of the human APP gene with the Swedish familial AD mutation, which was subsequently introduced into three different inbred mouse strains, namely C57BL/6J (termed B6-R1.40), 129S1/SvImJ (termed 129S1-R1.40) and DBA/2J (termed D2-R1.40) (Kulnane and Lamb, 2001; Lamb et al., 1999; Lamb et al.,
1997; Lamb et al., 1993; Lehman et al., 2003a; Lehman et al., 2003b). For the current studies, B6-R1.40, 129S1-R1.40 and D2-R1.40 animals were placed on the two defined low-fat/low-cholesterol (LF/LC) and high-fat/high-cholesterol (HF/HC) Clinton-Cybulsky diets (Lichtman et al., 1999). As expected, body weight and serum cholesterol levels were elevated in all three strains. However, only the B6-R1.40 mice exhibited increases in steady state brain Aβ levels that correlated with the levels of serum cholesterol. Somewhat surprisingly, the elevation in brain Aβ was not correlated with alterations in holo-APP levels or APP processing products nor steady state levels of APOE. Notably, the gene expression profile in the B6-R1.40 animals differed from the 129S1-R1.40 and D2-R1.40 strains. Our results suggest that there is a unique interaction between a postulated environmental risk factor for AD, namely diet and genetic background, which may have implications for the study of gene-environment interactions in human AD.

MATERIALS AND METHODS

Animals

The R1.40 transgene is a complete, full-length copy of the human APP gene (the 290 kb APP gene carried on a 650 kb yeast artificial chromosome) containing the early onset familial AD mutation K670N/M671L. Creation of the R1.40 transgenic mouse strain and subsequent backcrossing to the C57BL/6J (The Jackson Laboratory stock number 000664), DBA/2J (stock number 000671) and 129S1/SvImJ (stock number 002448) strains to generate the B6.129-
Tg(APPSw)40Btla/J (B6-R1.40), D2.129(B6)-Tg(APPSw)40Btla/J (D2-R1.40), and 129S1.129(Yamin et al., 1999)-Tg(APPSw)40Btla/J (129S1-R1.40) lines was described previously (Lamb et al., 1997; Lamb et al., 1993; Lehman et al., 2003a). Age- and gender-matched, twenty-eight day old hemizygous transgenic animals on the all three genetic backgrounds were divided into LF/LC or HF/HC fed groups. Animals were housed at the Cleveland Clinic Biological Resource Unit, a facility that is fully accredited by the Association of Assessment and Accreditation of Laboratory Animal Care. All animals were housed in ventilated micro-isolators maintained at 22°C on 14:10 h light:dark cycles, with food and water provided *ad libitum*. The Institutional Animal Care and Use Committee of the Cleveland Clinic Foundation approved all of the animal procedures.

**Diets**

The defined Clinton-Cybulsky diets were obtained from Research Diets Inc (Lichtman et al., 1999). The LF/LC group of mice was placed on cholesterol-free chow containing 10% of the kcal derived from fat (Catalog #D12102, Research Diets, Inc.), while the HF/HC group of mice was placed on a high-fat/high-cholesterol diet containing 1.25% cholesterol (gm % w/w) and 40% of the kcal derived from fat (Catalog #D12108, Research Diets, Inc) for a total of eight weeks. Body weight was measured every week and just prior to sacrifice on a calibrated scale.
Sample Collection

Tissue and serum were harvested from 16-22 animals per experimental group. Serum was collected via retro-orbital sinus bleeding using heparinized capillary tubes. Animals were fasted for 24 h prior to serum collection (fasted group), then exposed to diet for 24 h and bled again (fed group). Whole blood was centrifuged at 2200 x g for 5 min and the serum collected. Following cervical dislocation, brains were removed, bisected along the midline into the right and left hemispheres.

Measurement of Serum Cholesterol

Brain and serum cholesterol levels were measured using the Infinity Cholesterol Reagent as recommended by manufacturer (Thermo Scientific). The levels of brain cholesterol was determined on samples homogenized in 6 M urea buffer (6 M urea, 100 mM Tris pH 7.4, 1 mM DTT, 1 mM EDTA, 0.5 M AEBSF and 1% SDS) as described previously (Levin-Allerhand et al., 2002). Brain homogenates were centrifuged at 9000 x g for 10 min at 4°C, prior to measurement of cholesterol levels. Both brain supernatants and serum were diluted 1:1 with dH2O immediately prior to the measurement of cholesterol levels. Samples were incubated in the Thermotrace reagent for 10 min and the resulting chromophore (quinoneimine dye) was read at 490 nm as per the manufacturers instructions. Brain cholesterol levels were normalized to the total amount of protein in each sample as determined by the BCA (bicinchoninic acid) assay (Pierce) according to manufacturers instructions.
**Aβ ELISA**

Steady state brain Aβ levels were determined utilizing a human-specific fluorescent Aβ<sub>1-40</sub> ELISA (Biosource International) according to the manufacturer's instructions. Briefly, the left hemisphere was homogenized in 5 M guanidine–100 mM Tris pH 8.0 (1:8) and incubated at room temperature for 4 h with constant agitation to ensure complete protein extraction. Samples were diluted (1:20) in standard/sample buffer and centrifuged at 16,000 x g for 20 min at 4°C before being loaded in triplicate onto the ELISA plate for subsequent incubation with antibodies and fluorescent detection.

**Western Blots**

6 M urea brain extracts were sonicated using Sonic Dismembrator Model 500 (Fisher Scientific) for 20 sec at 20% amplitude to break up cellular DNA and subsequently centrifuged at 18000 x g for 30 min at 4°C. The resulting supernatant was diluted 1:4 with Tris-buffered saline (TBS) and protein concentration determined using the BCA protein assay kit (Therma Fisher Scientific). 20 µg (for APOE Western blots) or 50 µg (for CT15 Western blots) of brain protein was resolved on gradient 4-12% Bis-Tris NuPAGE gels (Invitrogen) and transferred to PVDF membrane (Millipore). Membranes were blocked in 5% milk in phosphate buffered saline (PBS, for APOE Western blots) or NAP<sub>TM</sub> Blocker (G-Biosciences, for CT15 Western blots) and incubated with a 1:100 dilution of an anti-human APOE antibody (Millipore, Cat # AB947) or a 1:5000
dilution of CT15, an antibody that recognizes the last 15 amino acids of mouse/human App/APP (Soriano et al., 2001) overnight at 4°C. Following 5 x 5 min washes in either PBS (for APOE Western blots) or TBS-0.01% Tween (for CT15 Western blots), membranes were incubated with either a horse-radish peroxidase (HRP) conjugated anti-goat secondary antibody for the APOE blots (Jackson ImmunoResearch Laboratories, catalog# 305-035-003), an HRP conjugated anti-rabbit secondary antibody for the CT15 blots (Jackson ImmunoResearch Laboratories, catalog #111-035-003,) or an HRP conjugated anti-mouse secondary antibody for βActin and GAPDH (Jackson ImmunoResearch Laboratories, catalog #115-035-003) at a 1:20,000 dilution in 5% milk (APOE, GAPDH) or NAP block (CT15, βActin). Following 5 x 5 min washes, the blots were incubated with Western Lightening™ Plus-ECL (Perkin Elmer) and exposed to X-ray film for multiple different exposure lengths to ensure detection of both full-length APP and APP CTFs within the linear range. Bands were quantified utilizing the AlphaEaseFC™ Software (Alpha Innotech Corporation). Relative levels of mouse APOE were quantified by APOE/GAPDH ratios in both the HF/HC and LF/LC fed animal. The relative level of holo-APP within each sample was measured via holo-APP/βActin ratio which was then used to determine APP CTF_β and CTF_α levels (CTF_β/holo-APP, CTF_α/holo-APP respectively).

Illumina Beadchip Microarray Analysis

Brains were homogenized with 2 ml of TRI REAGENT® (Sigma)
according to the manufacturer’s protocol. DNA was removed by TURBO DNA-
free™ (Ambion) according to manufacturers protocol. 250 ng of RNA was reverse
transcribed into cRNA and biotin-UTP labeled using the Illumina TotalPrep RNA
amplification kit (Ambion). cRNA was quantified using a nanodrop
spectrophotometer, and the cRNA quality (size distribution) was further analyzed
on a 1% agarose gel. cRNA was hybridized to the Illumina MouseRef8 v1.1
Expression BeadChip using standard protocols (provided by Illumina). Using
BeadStudio v3.0., raw data was background subtracted and normalized using the
quantile algorithm. Normalized data was subsequently filtered for \( p<0.05 \) and >2
fold changes in gene expression.

Quantitative Reverse Transcriptase PCR

The levels of 2 transcripts exhibiting alterations in the levels between
LF/LC and HF/HC samples were assayed utilizing TaqMan® Gene Expression
Assays Mm01195581_g1 (Hspb1) and Mm00446719_m1 (Tf) per the
manufacturer’s instructions. 50 ng RNA was used to generate cDNA via the
High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according
to manufacturers protocol. The cDNA was amplified with an adapted protocol
which combined 12.5 \( \mu l \) of the Taqman Universal PCR Master Mix (Applied
Biosystems) with 2.5 \( \mu l \) cDNA, 8.75 \( \mu l \) Nuclease-free water and 1.25 \( \mu l \) Taqman
Gene Expression Assay mix, specific for each gene (Applied Biosystems), for
each sample. Each sample was run in triplicate on the 7300 Real Time PCR
System (Applied Biosystems) and quantified using the Relative Quantification
Plate Document. For each Taqman probe, serial dilutions of total RNA were utilized to ensure that each probe was within the linear range. The threshold cycle (C_T) value was determined for each experimental sample and the glyceraldehyde 3 phosphate dehydrogenase (Gapdh, Illumina part number 4352932E) endogenous control was used to calculate the fold change ($2^{-\Delta\Delta CT}$).

**Statistical Analysis**

Body weight, serum cholesterol, and brain Aβ levels between LF/LC and HF/HC in all groups were compared using an one-way ANOVA of variance with a Bonferroni correction for multiple comparisons. An unpaired T-test was used to compare male and females within each group. A correlation was used to determine the relationship between two group, such as body weight/Aβ and cholesterol/Aβ. ANOVA, t-test and correlation were conducted using GraphPad PRISM. Microarray data was analyzed using BeadStudio v3.0., raw data was background subtracted and normalized using the quantile algorithm. Normalized data was subsequently filtered for p<0.05 and >2 fold changes in gene expression.

**RESULTS**

The congenic genomic-based R1.40 mouse model of AD was used to examine the effect of LF/LC versus HF/HC diets on body weight, serum and brain cholesterol, levels of APP and its processing products as well as steady state levels of brain Aβ. The R1.40 model contains four to six intact copies of an
APP yeast artificial chromosome (YAC) with the K670N/M671L early-onset familial AD mutation and expresses human APP mRNA and protein at levels two- to three-fold above endogenous App in the homozygous state (Lamb et al., 1997). R1.40 mice express all of the different isoforms of human APP in a tissue and cell-type specific manner (Lamb et al., 1993; Lehman et al., 2003b). In the C57BL/6J genetic background, R1.40 mice express high levels of brain Aβ and develop Aβ deposits beginning at 12 months of age and exhibit numerous biochemical, neuropathological, ultrastructural and behavioral similarities to human AD (Bhaskar et al., 2009; Hock, 2009; Kulnane and Lamb, 2001; Lamb et al., 1999; Lehman et al., 2003a; Lehman et al., 2003b; Varvel et al., 2008; Yang et al., 2006).

As a means to identify genetic modifiers of APP processing and Aβ metabolism in mice, the R1.40 YAC transgene was transferred into three different genetic backgrounds, B6-R1.40, D2-R1.40 and 129S1-R1.40 by repeated backcrossing to the corresponding inbred mouse strains for over 10 generations (Lehman et al., 2003a). Our previous results demonstrated that all three strains produce nearly identical levels of steady state holo-APP as well as the two primary APP C-terminal fragments, CTFα and CTFβ at young ages. Notably, however, the steady state levels of brain and plasma Aβ are dependent upon genetic background with highest levels in the B6-R1.40, lowest in D2-R1.40 and intermediate in the 129S1-R1.40 animals (Lehman et al., 2003a).
**Effects of Diet on Body Weight**

Dietary factors postulated as a risk factor for AD were examined to determine if genetic background altered the effects of diet on body weight. B6-R1.40, D2-R1.40 and 129S1-R1.40 animals were placed on the defined Clinton-Cybulsky diets at weaning for a total of eight weeks. The Clinton-Cybulsky diets are companion diets that have either 10% Kcal from fat/0% cholesterol (LF/LC) or 40% Kcal from fat/1.25% cholesterol (HF/HC) (Lichtman et al., 1999). As expected from data in the Mouse Phenome Database (http://www.jax.org/phenome), all three of the R1.40 strains on the HF/HC diet exhibited increases in average body weight when compared to animals maintained on the companion LF/LC diet (Figure 2-1A). Specifically, B6-R1.40 mice on the HF/HC diet had an average body weight of 24.88 g compared to 22.20 g on the LF/LC diet; D2-R1.40 mice on the HF/HC diet had an average body weight of 30.32 g compared to 24.15 g on the LF/LC diet; and 129S1-R1.40 mice on the HF/HC diet had an average body weight of 25.18 g compared to 23.51 g on the LF/LC diet. While all groups exhibited an apparent increase in body weight on the HF/HC diet, only the D2-R1.40 animals exhibit a difference that was statistically significant (p<0.001 one-way ANOVA of variance with the Bonferroni correction for multiple comparisons). For all strains on both the HF/HC and LF/LC diets the males weighed significantly more than the females (p<0.0001) (Supplemental Figure 2-1).
**Effects of Diet on Cholesterol**

The effect of the LF/LC and HF/HC diets on serum cholesterol levels was then examined in the B6-R1.40, D2-R1.40 and 129S1.1R1.40 animals using serum isolated from both fasted or fed animals. All three strains exhibited a statistically significant elevation in the levels of fasted and fed serum cholesterol on the HF/HC diet when compared to the LF/LC diet (Figure 2-1B and C). For example, B6-R1.40 mice on a HF/HC diet had average fasted serum cholesterol levels of 154.9 mg/dL, while mice on the LF/LC diet had fasted serum cholesterol levels of 88.79 mg/dL (p<0.001); D2-R1.40 mice on the HF/HC diet had fasted serum cholesterol levels of 128.0 mg/dL, while mice on the LF/LC diet had fasted serum cholesterol levels of 84.19 mg/dL (p<0.001); and 129S1-R1.40 mice on the HF/HC diet had fasted serum cholesterol levels of 182.2 mg/dL, while mice on the LF/LC diet had fasted serum cholesterol levels of 142.0 mg/dL (p<0.001). These results are similar to what has been reported for the C57BL/6J, DBA/2J and 129S1/SvImJ parental strains. Also similar to data in the literature and the Mouse Phenome Database (http://www.jax.org/phenome), across all groups males had significantly higher cholesterol levels than females (p<0.05) (Svenson et al., 2007).

Next, to determine whether the LF/LC and HF/HC diets had a similar effect on total levels of brain cholesterol in the B6-R1.40 D2-R1.40 and 129S1-R1.40 mice, hemi-brains were extracted and total cholesterol measured (Supplemental Figure 2-2). Total brain cholesterol levels were elevated in the HF/HC fed animals for all strains, however it did not reach significance.
Effects of Diet on Aβ Metabolism

Finally, dietary factors that influenced body weight and serum cholesterol levels were tested to see if they influenced brain Aβ metabolism. The levels of total steady state brain Aβ was measured by an Aβ1-40 ELISAs in B6-R1.40, D2-R1.40 and 129S1-R1.40 mice on both LF/LC and HF/HC diets (Figure 2-1D). B6-R1.40 animals on the HF/HC diet exhibited the highest average level of steady state brain Aβ at 6.51 pmol/g (of brain tissue), which was significantly higher than B6-R1.40 animals on the LF/LC diet at 5.05 pmol/g of Aβ (p<0.001). By contrast, D2-R1.40 mice on the HF/HC diet exhibited lower levels of brain Aβ at 4.08 pmol/g, which was not significantly different from D2-R1.40 animals on the LF/LC diet at 4.14 pmol/g (p>0.05). 129S1-R1.40 mice also did not exhibit a statistically significant difference in brain Aβ levels, as animals on the HF/HC diet exhibited average brain Aβ levels of 4.83 pmol/g and animals on the LF/LC diet exhibited average brain Aβ levels of 5.18 pmol/g. Interestingly, brain Aβ levels were not influenced by gender, male and female Aβ levels were not significantly different in any of the groups (p>0.05). Thus, although body weight and serum cholesterol increased in all three R1.40 strains on the HF/HC diets, the steady state levels of brain Aβ increased only in the B6-R1.40 strain.

Genetic Background Dependent Correlation Between Serum Cholesterol and Aβ Levels

To determine whether there was a relationship between serum cholesterol
and body weight or brain Aβ levels, a correlational analysis was performed within the different groups of animals (Figure 2-2). Measurements of body weight did not correlate with steady state brain Aβ levels in all strains (Figure 2-2A: LF/LC, HF/HC, all strains \([R^2=0.03]\)). By contrast, the levels of brain Aβ correlated with fed serum cholesterol levels in all strains (Figure 2-2B) showed a slight correlation, \(R^2=0.17\), due to the strong correlation in the B6-R1.40 strain, \(R^2=0.42\) (Figure 2-2D). Interestingly, neither the D2-R1.40 (\(R^2=0.04\)) nor 129S1-R1.40 (\(R^2=0.007\)) animals had a correlation between serum cholesterol and brain Aβ levels (Figure 2-2C and E). The same trend was observed in correlational analysis of brain Aβ levels and fasted serum cholesterol (Supplemental Figure 2-4). This data suggested that there was a genetic background dependent relationship between the elevation in serum cholesterol and brain Aβ.

**Effects of Diet on APP Expression and APP Processing**

One possible explanation for the increase in brain Aβ levels in the B6-R1.40 animals on the HF/HC diet was that there was a genetic background dependent increase in APP expression. To examine this, Western blot analysis was performed on total protein brain extracts from B6-R1.40 animals on LF/LC and HF/HC diets utilizing the antibody CT15, which recognizes the final 15 amino acids of APP. Quantification of full-length APP revealed that there was no significant difference in the levels of the 95 kDa holo-APP between the LF/LC and HF/HC diets (Figures 2-3A and B).

Previous studies both *in vitro* and *in vivo* suggested that alterations in
cholesterol influences the cleavage of APP into CTF$_{\beta}$, the immediate precursor of A$\beta$, by the primary $\beta$ secretase, BACE1 [reviewed in (Carter, 2007)]. To determine if the processing of APP into the different APP CTFs could explain the differences in A$\beta$ levels between B6-R1.40 animals on the LF/LC and HF/HC diets, the steady state brain levels of APP CTF$_{\alpha}$ and CTF$_{\beta}$ was determined by Western blot analysis using the CT15 antibody. Quantification of the amount of CTF$_{\alpha}$ (expressed as the ratio CTF$_{\alpha}$/ holo-APP) and CTF$_{\beta}$ (expressed as the ratio of CTF$_{\beta}$/ holo-APP), revealed that there were no significant alterations in either APP processing product between B6-R.140 animals on the LF/LC and HF/HC diets (Figures 2-3C and D). Our results suggest that the effects of HF/HC diets on the B6-R1.40 strain were not mediated by altered processing towards the amyloidogenic pathway.

**Effects of Diet on Apoe Expression**

Another potential explanation for the correlation between serum cholesterol and brain A$\beta$ levels in animals on the LF/LC and HF/HC diets in the B6-R1.40 mice, was altered expression of brain Apoe. APOE is the primary cholesterol transporter in the brain and has been implicated in various A$\beta$ clearance pathways and $APOE$ expression levels are regulated by cholesterol metabolism (Deane et al., 2008; Mahley, 1988; Srivastava, 1996; Strittmatter et al., 1993). Western blot analysis of brain extracts from B6-R1.40 mice on LF/LC and HF/HC diets with an antibody against APOE, revealed that there were no statistically significant ($p>0.08$) alterations in APOE levels (Figure 2-4A and B).
Effects of Diet on Brain Gene Expression

To further examine brain-specific alterations that might be responsible for the correlation between serum cholesterol and brain Aβ in B6-R1.40, but not the 129S1-R1.40 and D2-R1.40 animals on the LF/LC and HF/HC diets, gene expression microarray analysis was performed using the Illumina MouseRef-8 BeadChip. The MouseRef-8 BeadChip enables the genome-wide analysis of gene expression on 25,600 well-annotated genes from the National Center for Biotechnology Information Reference Sequence (NCBI RefSeq) database (Build 36, Release 22) using Illumina’s BeadArray technology. Total brain RNA from B6-R.140, 129S1-R1.40 and D2-R1.40 animals on the LF/LC and HF/HC diets for 8 weeks was isolated, reverse transcribed and hybridized to the MouseRef-8 BeadChips and analyzed on the Illumina Beadstation. Gene expression data for the >19,000 ReSeq genes was filtered for genome-wide statistical significance (p<0.05) and >2-fold differences between the LF/LC and HF/HC groups for each strain.

Notably, B6-R1.40 animals exhibited 159 genes that met the established criteria for altered expression between the LF/LC and HF/HC groups (Supplemental Table 2-1). By contrast, the D2-R1.40 animals did not exhibit any genes that met the criteria between the LF/LC and HF/HC groups, while 129S1-R1.40 animals exhibited only six genes that met the criteria (Supplemental Table 2-2). One of the six genes (Junb, a transcription factor) exhibiting altered expression in the 129S1-R.140 animals was also altered in the
B6-R1.40 animals. When the data was plotted in a heat map, the B6-R1.40 animals on the LF/LC diet were considerably different than either the B6-R1.40 animals on the HF/HC diet or the D2-R1.40 and 129S1-R1.40 animals on either the LF/LC or HF/HC groups (Figure 2-5). This data suggests that B6-R1.40 animals on a LF/LC diet exhibit a unique gene expression profile that is specifically altered when animals were placed on a HF/HC diet and correlates with the elevation in brain Aβ levels.

Pathway analysis of the 159 genes in the B6-R.140 HF/HC fed animals, using Ingenuity pathway analysis software (www.ingenuity.com), revealed an association of many of the genes with neurological disease as well as nervous system development and function. Within this group of genes there were those associated with (see Table 2-1); altered APP processing/Aβ metabolism [Thop1 (Pollio et al., 2008; Yamin et al., 1999), Rab1b (Dugan et al., 1995) and Tspan12 (Xu et al., 2009)], altered risk for AD [Fgf1 (Yamagata et al., 2004), Hsd11b1 (de Quervain et al., 2004; Deary et al., 2006) and Tf (Namekata et al., 1997; Van Landeghem et al., 1998; van Rensburg et al., 1993)], interactions with Aβ [Hspb1 (Wilhelmus et al., 2006; Yoshiike et al., 2008)] and a role in atherosclerosis and/or type 2 diabetes [Gja4 (Wong et al., 2006), Dcn (Al Haj Zen et al., 2006), Hsd11b1 (Franks et al., 2004; Masuzaki et al., 2001) and Tcf7l2 (Huertas-Vazquez et al., 2008; Thorsby et al., 2009; Tong et al., 2009; Yan et al., 2009)] (Table 2-1).

To confirm the gene expression microarray findings, two of these genes, one with increased (Tf) and one with decreased (Hspb1) expression between the
B6-R.140 mice on the LF/LC and HF/HC diets were chosen and analyzed via quantitative reverse transcriptase-PCR (qRT-PCR) using specific TaqMan probes. As expected, and similar to the microarray data, qRT-PCR revealed the same trend, *Tf* had a significant increase in gene expression (p=0.02, unpaired t-test) and *Hspb1* had a significant decrease in gene expression (p=0.009, unpaired t-test) (Figure 2-6).

**DISCUSSION**

Increasing evidence suggests that AD is likely triggered by a complex interplay between both genetic and environmental risk factors. Identification of the genetic factors responsible for altered AD risk has proven exceedingly difficult, likely due to genetic and phenotypic heterogeneity, small effect sizes and influence of environmental factors. Similarly, while there is considerable epidemiological data in support of various environmental risk factors in AD, few prospective studies have attempted to examine the role of specific environmental interventions or the interplay between specific genetic and environmental factors.

Most of the genetic studies of AD to date have supported a role of altered Aβ metabolism in the pathogenesis of AD. This includes increased APP gene dosage results in early-onset familial AD and AD-like features observed in Down syndrome, autosomal dominant mutations in APP that results in early-onset familial AD, and autosomal dominant mutations in *presenilin 1*, the gene encoding the catalytic subunit of the enzyme that generates Aβ, that also results in early-onset familial AD (Prasher et al., 1998; Rovelet-Lecrux et al., 2006; Tanzi...
and Bertram, 2001). In the present study, the effects of a postulated environmental risk factor for AD, namely HF/HC diets, on genetic background-dependent alterations in brain Aβ metabolism were examined using the R1.40 transgenic model of AD in the C57BL/6J, DBA/2J and 129S1/SvImJ mouse strains. Our results demonstrate that while HF/HC diets increased body weight and induced hypercholesterolemia in the B6-R1.40, D2-R1.40 and 129S1-R1.40 mouse strains, brain Aβ levels increased in the B6-R1.40, but not D2-R1.40 and 129S1-R1.40 mice that correlated with a unique diet-dependent brain gene expression profile exhibited by the B6-R1.40 mice that has direct implications for studies of gene-environment interactions in AD phenotypes as outlined below.

First, our results demonstrate that HF/HC diets induce hypercholesterolemia in all three congenic R1.40 mouse models of AD, but increase brain Aβ levels only in the B6-R1.40 mice. Not surprisingly, of the previously published studies examining the effects of high-fat/high-cholesterol diets on transgenic mouse models of AD, all but one reported increases in serum cholesterol levels (Fitz et al., 2010; George et al., 2004; Hooijmans et al., 2007; Howland et al., 1998; Levin-Allerhand et al., 2002; Li et al., 2003; Refolo et al., 2000; Shie et al., 2002). The exception was a study examining the effects of a high-fat diet in the absence of added cholesterol and in the presence of reduced carbohydrates resulting in a ketogenic diet (Van der Auwera et al., 2005).

By contrast, the previously published studies of the effects of high-fat/high cholesterol diets on brain Aβ levels are inconsistent. Several studies have shown that diet-induced hypercholesterolemia significantly increases brain Aβ
levels, based on Aβ ELISA or Aβ deposition (Fitz et al., 2010; Hoojimans et al., 2007; Levin-Allerhand et al., 2002; Li et al., 2003; Pedrini et al., 2009; Refolo et al., 2000; Shie et al., 2002). By contrast, other studies have shown that a high-fat/high-cholesterol diet can actually reduce levels of brain Aβ (George et al., 2004; Howland et al., 1998; Van der Auwera et al., 2005). The difference between the effects on HF/HC diets on brain Aβ levels in the published studies and the present study could be due to differences in the diets, promoters, transgenes, inclusion of different familial AD mutations or genetic background of the animals. Notably, and in support of the possible contribution of genetic background for the differences in the observed results from the studies, all of the published studies conducted in AD mice in the inbred C57BL/6 background have been report to lead to increased brain Aβ levels (Fitz et al., 2010; Hoojimans et al., 2007; Levin-Allerhand et al., 2002; Li et al., 2003). While none of the published studies utilized the DBA/2J or 129S1/SvImJ inbred mouse strains, of the two studies that observed decreases in brain Aβ levels, one study was conducted in AD transgenic mice maintained by interbreeding heterozygous animals on the CD-1 outbred genetic background, while the other utilized the ketogenic diet in AD mice maintained in the FVB inbred genetic background (Howland et al., 1998; Van der Auwera et al., 2005).

Second, the elevation in brain Aβ levels induced by the HF/HC diet is not correlated with altered APP expression or processing in the B6-R1.40 mice. While the mechanisms by which cholesterol can modify brain Aβ levels is not completely understood, cholesterol is found in lipid rafts along with APP, Aβ, the
γ-secretase component presenilin-1 and β-secretase (BACE1) and is thought to mediate amyloidogenic processing of APP through either modification of subcellular localization or facilitation of interactions between the proteases and their substrates (Cordy et al., 2003; Guardia-Laguarta et al., 2009; Riddell et al., 2001; Tun et al., 2002; Wahrle et al., 2002). Biochemical studies utilizing primary neurons and peripheral cells have demonstrated that cellular cholesterol content is correlated with Aβ generation and that this effect may be mediated through alterations in the β site cleavage of APP (Fassbender et al., 2001; Frears et al., 1999; Kojro et al., 2001; Ostrowski et al., 2007; Simons et al., 1998). However, the data from various animal models of AD in studies of dietary, genetic and therapeutic modulation of cholesterol metabolism have mixed results with regard to the relationship between serum cholesterol levels and APP processing. One study, using a transgenic mouse model of AD with both APP and PSEN1 familial AD mutations on a mixed genetic background, demonstrated that a high-fat/high-cholesterol diet with sodium cholate resulted in elevation of serum cholesterol, brain Aβ, and APP CTFβ, as well as a reduction in APPsα (Refolo et al., 2000). Two studies, one utilizing the Tg2576 mouse on a F2 genetic background and the other using a knock-in APP mouse model of AD in an the outbred strain CD-1 demonstrated that high-fat/high-cholesterol diets lead to decreases in secreted APP derivative APPsα, with no detectable alterations in cell-associated APP or APP CTFs (Howland et al., 1998). Two studies in two different transgenic models on the C57BL/6J genetic background showed no change in APP processing (Fitz et al., 2010; Levin-Allerhand et al., 2002). While most of the
animal studies show decreases in APPs\textsubscript{\alpha}, none showed increases in APPs\textsubscript{\beta} and only one study showed changes in CTFs. The reasons for the apparent discrepancy is unclear it may be because each animal study utilized different diets, had different familial AD mutations and were on different genetic backgrounds. Our lab has shown previously that APP processing varies between mouse models, particularly with respect to brain region, transgene and genetic background and that steady state levels of APP CTFs were not correlated with the levels if A\textsubscript{\beta} (Lehman el al., 2003a; Lehman et al., 2003b). The current studies provide further support that the effects of HF/HC diets on A\textsubscript{\beta} metabolism is independent of effects on steady state levels of APP CTFs.

Third, the strain-specific effects of HF/HC diets on brain A\textsubscript{\beta} is not correlated with alterations in steady state levels of APOE. APOE is the primary cholesterol transporter within the brain, can directly bind to A\textsubscript{\beta} and has been implicated in the clearance of A\textsubscript{\beta} from the brain (Deane et al., 2008; Mahley, 1988; Strittmatter et al., 1993). Common APOE polymorphisms in humans are linked to increased risk for AD, with the \(\epsilon4\) allele conferring highest risk (Corder et al., 1993). Interestingly, human studies reveal that in individuals with the same APOE \(\epsilon4\) allele, the association with AD risk varies based on race, the association is higher in Japanese subjects compared to Caucasians and even weaker in African-American and Hispanic populations, showing that susceptibility alleles within certain populations can modify the effects of APOE (Farrer et al., 1997). Analysis of gene expression profiles of brain Apoe levels in inbred mouse strains using Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) did
not reveal significant variation in Apoe levels between strains (Fernandes et al., 2004; Hovatta et al., 2005; Hovatta et al., 2007). However, when looking at the effects of hypercholesterolemia in mouse models of AD, several studies have observed associations with brain APOE. In three studies, the levels of brain APOE directly correlated with the levels of Aβ, (Fitz et al., 2010; Levin-Allerhand et al., 2002; Shie et al., 2002) while in one study there was an inverse correlation (Howland et al., 1998). Notably, in the studies presented here, no increase in brain APOE levels was observed in animals on the HF/HC diets, suggesting that the effects on Aβ levels in the B6-R1.40 strain was not due to alterations in APOE levels in this genetic background.

Fourth, B6-R1.40 animals on the LF/LC and HF/HC diet exhibited a unique brain gene expression profile between experimental groups within the same strain that was not observed in the D2-R1.40 and 129S1-R1.40 animals. In particular, there were 159 genes differentially expressed in the B6-R1.40 mice, only six genes were differentially regulated in the 129S1-R1.40 mice and no genes were differentially expressed in the D2-R1.40 mice. Surprisingly, analysis of the genes differentially expressed in B6-R1.40 mice did not reveal altered regulation of genes in the cholesterol pathway, including Apoe (which was consistent with the lack of an alteration in Apoe protein levels reported in this study). In addition, none of the genes previously implicated in App expression and processing were altered. This includes; App and its paralogs Aplp1 and Aplp2; the metalloproteases Adam9, Adam10 and Adam17 all implicated in the α secretase processing of App; Aph, Ncstn, Pen2, Psen1 and Psen2, subunits of
the γ secretase; the aspartyl proteases \textit{Bace1} and \textit{Bace2}, the primary β secretases; the Aβ degrading proteases \textit{Ide} and \textit{Mme} (Neprilysin); and \textit{Ager} (Receptor for advanced glycosylation end product), \textit{Lrp1} and \textit{Apoe}, all implicated in Aβ clearance mechanisms.

Closer examination of the 159 genes differentially expressed revealed several with potential links to APP processing, Aβ metabolism and AD pathogenesis. First, several of the genes have been implicated in altered APP processing and Aβ metabolism: \textit{tetraspanin 12}, \textit{Tspan12} (Postina et al., 2004; Xu et al., 2009), \textit{ras-related GTP-binding protein}, \textit{Rab1b} (Dugan et al., 1995) and \textit{thimet oligopeptidase 1}, \textit{Thop1} (Pollio et al., 2008; Yamin et al., 1999). \textit{Thop1}, a metalloprotease that cleaves small peptides, has been shown \textit{in vitro} to cleave Aβ and provide protection from Aβ toxicity. It is also up regulated in the AD brain.

Second, two \textit{heat shock protein} (\textit{Hsp}) genes were downregulated in B6-R1.40 animals on the HF/HC diet, \textit{heat shock 27 kDa protein 1}, \textit{Hspb1} and \textit{heat shock 70 kDa protein 5}, \textit{Hspa5}. HSPs are highly regulated proteins involved in normal cellular activity. They become upregulated upon cellular exposure to stress. Functioning as molecular chaperones, HSPs mediate proper protein folding and promote recovery of native protein conformation lost due to stress. Improperly folded or denatured proteins aggregate and accumulate in cells. Although the mechanism by which HSPs are involved in AD is not completely understood, they have been associated with AD in several ways. Studies have shown levels of HSPs are increased in amnestic mild cognitive impairment
(aMCI), vascular mild cognitive impairment (vMCI) and AD (Bjorkdahl et al., 2008; Di Domenico et al., 2010; Lee et al., 2008). HSPs have also been shown to bind Aβ oligomers as well as co-localize with Aβ deposits in human AD brains (Wilhelms et al., 2006; Yoshiike et al., 2008). Finally, in vitro experiments have shown associations with HSPs and pathological hallmarks of AD. Aβ treatment of primary neurons increases HSPs and cells treated with HSPs increase tau phosphorylation (Bjorkdahl et al., 2008; Yang et al., 2009). Likewise, overexpression of HSPs also increases the number of G(1)phase cells perhaps contributing to the cell cycle reentry seen in AD (Bjorkdahl et al., 2008).

Third, several genes exhibiting altered expression on HF/HC diets in the B6-R1.40 animals have also been implicated in human genetic association studies of AD. This includes polymorphisms in the fibroblast growth factor 1, Fgf1 (Yamagata et al., 2004), hydroxysteroid (11-β) dehydrogenase 1, Hsd11b1 (de Quervain et al., 2004; Deary et al., 2006) and transferrin, Tf (Mainous et al., 2005; Namekata et al., 1997; Van Landeghem et al., 1998; van Rensburg et al., 1993) genes. TF encodes an iron transport protein and studies have implicated altered iron metabolism in AD pathogenesis (Lehmann et al., 2006). TF has been specifically linked to AD in genetic association studies where unique polymorphisms in TF are associated with increased risk for developing AD (Bertram et al., 2007). These genetic associations between polymorphisms in TF and AD have been replicated in several independent studies and meta-analysis indicates a small, but significant association between the C2 allele at SNP rs1049296 with AD (Bertram et al., 2007). Transferrin and AD have also been
linked experimentally. Elevation in cholesterol and transferrin saturation, a marker of the body’s iron stores, have been correlated to increased AD risk (Namekata et al., 1997; Van Landeghem et al., 1998; van Rensburg et al., 1993) and transferrin staining has been observed around senile plaques in the brains of AD patients (Connor et al., 1992). The fact that Tf is upregulated in the HF/HC fed B6-R1.40 animals suggests that it could contribute to increased levels of Aβ observed.

Finally, several genes with altered expression in the HF/HC diet group have been implicated in atherosclerosis and/or type 2 diabetes, two diseases that exhibit substantial co-morbidity with AD. This includes the gap junction protein, alpha 4, Gja4 [also known as Cx37 (Wong et al., 2006)], decorin, Dcn (Al Haj Zen et al., 2006) and transcription factor 7-like 2, Tcf7l2 (Huertas-Vazquez et al., 2008; Thorsby et al., 2009; Tong et al., 2009; Yan et al., 2009) genes. However, the exact role these genes may play in AD is uncertain as only one study has examine the association between polymorphisms in the transcription factor 7-like 2 gene and AD and the results were negative (Figgins et al., 2009).

While the differential expression of these genes previously associated with AD as well as the rest of the 159 genes altered on the HF/HC diet are mechanistically involved in altering steady state Aβ levels, it is likely the interaction of these genes with susceptibility loci within the C57BL/6J strain that influence the animal’s risk to the Aβ elevating effects of a high-fat/high-cholesterol diet. A previous mapping study to identify genetic modifiers of brain Aβ in the congenic R1.40 mouse model of AD revealed that the difference in
steady state Aβ between the B6-R1.40 and D2-R1.40 strains are due to multiple genes, with each locus accounting for approximately 4% of the total variance (Ryman et al., 2008). This is likely also the case in this study, many genes with small effect size. The role of these genetic loci in regulating Aβ metabolism and deposition is currently being assessed using consomic and congenic strains in which individual chromosomes or chromosomal regions are carefully examined. It seems likely that understanding the mechanisms underlying the complex interplay between genes and environment will require similar approaches utilizing the sophisticated mouse genetic resources available.

The current studies provide the first experimental evidence that the effects of a postulated environmental risk factor for AD, namely dietary fat and cholesterol, is dependent upon genetic background. There is precedent for such complex gene-environment interactions in other common human diseases. For example, in the case of small vessel cerebrovascular disease, studies have shown that Japanese ancestry is associated with increased susceptibility for the disease. However, a westernized diet high in fat and protein compared to the traditional diet of mainland Japan reduces the risk for vascular dementia [reviewed in (Shadlen et al., 2002)]. Due to allelic variation in the Japanese race, a westernized diet traditionally associated with poor vascular health is actually protective. Likewise, allelic variation in the DBA2/J strain prevents changes in Aβ in the presence of HF/HC diet. Our current findings have implications for studies of AD pathogenesis using mouse models as well as ongoing genome-wide association studies of AD, in which environmental factors are difficult, if not
impossible, to control for. Thus, it seems likely that numerous environmental risk factors for AD may induce risk dependent upon the genetic factors specific to a particular population.
Figure 2-1: Genetic background dependent effects of HF/HC diet on body weight, serum cholesterol and brain Aβ levels. A-D, hemizygous R1.40 animals on three genetic backgrounds, DBA/2J, C57BL/6J, and 129S1/SvImJ, were fed a LF/LC or HF/HC diet for 8 weeks (n=16-22 animals per group). The trends for males and females were the same for all groups so all animals were combined. Error bars represent standard error of the mean. A, body weight prior to sacrifice. All strains showed an apparent increase in body weight on the HF/HC diet that reached statistical significance in the D2-R1.40 animals (p<0.001, one-way ANOVA of variance with the Bonferroni correction for multiple comparisons). For all strains on both diets, males had a significantly higher body weight than females (p<0.0001, unpaired t-test). B-C, cholesterol measurements from serum collected after animals were fasted for 24 hours (fasted) and again after exposure to diet for 24 hours (fed). The HF/HC fed D2-R1.40 animals had significantly higher fasted (p<0.001) and fed (p<0.05) serum cholesterol levels compared to LF/LC fed D2-R1.40 animals. Likewise, the HF/HC fed B6-R1.40 (p<0.001, fasted and fed) and 129S1-R1.40 (p<0.05, fasted and fed) animals had significantly higher levels than their LF/LC fed counterparts. Males had higher serum cholesterol levels than females (p<0.05). D, Guanidine-HCl-extracted brain homogenates were analyzed on Aβ ELISAs with Aβ standards and expressed as pmol Aβ/g brain tissue. Brain Aβ levels were significantly elevated exclusively in the B6-R1.40 HF/HC fed animals (p<0.001).
Figure 2-2: Correlation of brain Aβ levels with serum cholesterol in B6-R.140 mice. A, Aβ levels for all animals were correlated with body weight. The correlation coefficient was near zero, indicating that body weight is not correlated with brain Aβ levels ($R^2=0.03$). B, Aβ levels were correlated with serum cholesterol levels for all animals. There is a ($R^2=0.17$) correlation between brain Aβ and fed serum cholesterol when the entire group of animals is compared. C-E, Aβ levels were correlated with serum cholesterol levels within each strain. Notably, brain Aβ levels and fed serum cholesterol levels were correlated ($R^2=0.42$) only in the B6-R1.40 animals, D; not in the D2-R1.40 ($R^2=0.04$), C; or 129S1-R1.40 ($R^2=0.007$) animals, E. The trends for males and females were the same for all groups so all animals were combined. All trends were similar in fasted samples (Supplemental Figure 2-3).
Figure 2-3: Unaltered APP expression and processing in B6-R1.40 animals on the HF/HC diet. A, Western blot analysis of APP and APP CTF levels. 6 M urea extracted hemi-brain lysates from low-fat/low-cholesterol (B6-LF, n=3; lanes 1-3) and high-fat/high-cholesterol (B6-HF; lanes 4-6) fed animals were electrophoresed on 10-20% Bis-Tris gradient gels and blotted with the anti-APP C-terminal antibody CT15. Each lane was loaded with 50 µg protein. Quantification of the blots revealed no statistically significant differences in the levels of holo-APP (hAPP, B), CTF$_{\beta}$ (expressed as CTF$_{\beta}$/CTF$_{Total}$, C) or CTF$_{\alpha}$ (expressed at CTF$_{\alpha}$/CTF$_{Total}$, D) in B6-R1.40 animals in the LF/LC or HF/HC groups. Error bars represent standard error of the mean.
Figure 2-4: Similar brain Apoe levels in B6-R1.40 animals on the HF/HC and LF/LC diets. A, Western blot analysis of Apoe expression. 6 M urea extracted hemi-brain lysates from 12 weeks old LF/LC (B6-LF, n=5; lanes 1-5) and HF/HC (B6-HF, n=5; lanes 6-10) fed mice. Each lane was loaded with 50 µg of total protein. B, Quantification of the Western blots revealed no significant difference in the expression levels of brain Apoe (p>0.08) between B6-R.140 in the LF/LC and HF/HC groups. Error bars represent standard error of the mean.
Figure 2-5: Unique brain gene expression profile in B6-R1.40 mice on LF/LC and HF/HC diets. A, heat map of gene expression of the high-fat/high-cholesterol (HF/HC) and low-fat/low-cholesterol (LF/LC) fed animals in the B6-R1.40, D2-R1.40 and 129S1-R1.40 animals (n=3 per group). Included are the 159 genes that were differentially expressed between the HF/HC and LF/LC fed B6-R1.40 animals. Red represents genes with increased expression; yellow represents genes unchanged and green represents genes with decreased expression. Experimental groups are clustered based on their similarity in gene expression.
Figure 2-6: qRT-PCR verification of a subset of genes identified by microarray. RNA isolated from whole brains of 8 week old HF/HC and LF/LC fed B6-R1.40 animals (n=3-4 animals per group) were analyzed by quantitative real-time PCR analysis for gene expression of Tf and Hspb1. A, gene expression of Tf was significantly increased in HF fed B6-R1.40 animals compared to the LF fed B6-R1.40 animals (p=0.02; t-test). B, the Hspb1 gene had a statistically significant reduction in gene expression in HF fed B6-R1.40 animals compared to the LF fed B6-R1.40 animals (p=0.009, t-test). Error bars represent standard error of the mean.
Table 2-1: Genes exhibiting altered expression on HF/HC diets

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<tr>
<th>Genes Implicated In:</th>
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<th>Function</th>
<th>Change in Expression</th>
<th>Reference</th>
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<td>Thop1</td>
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Supplemental Figure 2-1: Gender differences in body weight on HF/HC diet.

Hemizygous R1.40 animals on three genetic backgrounds (DBA/2J, C57BL/6J, and 129S1/SvImJ) were fed LF/LC or HF/HC diet for 8 weeks and weighed prior to sacrifice (n=6-13 animals per group for females and n=7-11 animals per group for males). Error bars represent standard error of the mean. Female (A), all strains showed an increase in body weight on the HF/HC diet that reached statistical significance in the D2-R1.40 animals (p<0.001, one-way ANOVA of variance with the Bonferroni correction for multiple comparisons). Male (B), all strains showed a statistically significant increase in body weight on the HF/HC diet (p<0.001, D2-R1.40; p<0.05 B6-R1.40 and 129S1-R1.40).
Supplemental Figure 2-2: Genetic background dependant effect of HF/HC diet on brain cholesterol. Hemizygous R1.40 mice on three genetic backgrounds, DBA/2J, C57BL/6J and 129S1/SvImJ, were fed LF/LC or HF/HC diet for 8 weeks (n=5-12 animals per group). 6M urea whole brain extracts were analyzed using the Infinity Cholesterol reagent and were normalized to total protein within each sample. Error bars represent standard error of the mean. HF fed animals showed a slight elevation in brain cholesterol in all strains, however it did not reach significance.
Supplemental Figure 2-3: Correlation of brain Aβ levels with body weight in individual R1.40 lines. HF/HC and LF/LC fed 8 week old hemizygous R1.40 animals on 3 different inbred strain, DBA/2J, C57BL/6J, 129S1/SvImJ, were weighed prior to sacrifice and whole brain guanidine-HCl-extracts were analyzed on Aβ ELISAs for steady state Aβ levels. Statistical analysis of the two determined there was a correlation between brain Aβ and body weight in the B6-R1.40 animals but not the D2-R1.40 or 129S1-R1.40 animals (D2-R1.40 R²=0.02; B6-R1.40 R²=0.3; 129S1-R1.40 R²=0.03).
Supplemental Figure 2-4: Correlation of brain Aβ with fasted serum cholesterol in R1.40 animals. 8 week old hemizygous R1.40 animals on three different inbred strains, DBA/2J, C57BL/6J and 129SvImJ, were fed HF/HC or LF/LC diet (n=16-22 animals per group). Animals were fasted for 24 hours and serum cholesterol levels measured. These levels were correlated to Aβ levels ascertained from whole brain guanidine-HCl extracts using Aβ ELISA. The correlation coefficient was significant between brain Aβ and fasted serum cholesterol when the entire group of animals was compared (R²=0.09). This trend was due to the significant correlation of Aβ levels with fasted serum cholesterol in the B6-R1.40 animals (R²=0.40). Aβ levels were not correlated with fasted serum cholesterol levels within the D2-R1.40 (R²=0.05) and 129S1-R1.40 (R²=0.07) animals. The results are similar in fed animals (Figure 2-2)
### Supplemental Table 2-1: 159 genes differentially expressed in HF/HC fed B6-R1.40 animals.

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Chapter 3: The Role of the Complement Protein C5 Signaling in Beta-Amyloid Deposition

Stefanie Schrump¹,², Karen Mann¹, Guixiang Xu², and Bruce Lamb¹,²

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*In preparation
ABSTRACT

Alzheimer’s disease is a complex neurodegenerative disorder characterized by distinct neuropathological hallmarks including deposits of β-amyloid (Aβ) peptide. The genetics of early onset Alzheimer’s disease (AD) implicates the amyloid precursor protein (APP) and its proteolytic processing product, Aβ, as being central to disease pathogenesis. Previous studies using the R1.40 mouse model of AD have determined that genetic background has a profound effect on Aβ metabolism and deposition, with elevated steady state Aβ levels and robust Aβ deposition in the C57BL/6J inbred background and yet reduced Aβ levels and minimal Aβ deposition in the DBA/2J inbred background. Genetic mapping studies subsequently identified a locus on DBA/2J mouse chromosome 2 which is significantly associated with reduced levels of Aβ. In the current studies, the presence of a DBA/2J chromosome 2 locus was confirmed via the generation of a C57BL/6J congenic strain with a portion of DBA/2J chromosome 2 that reduces Aβ deposition in the R1.40 mouse model of AD. Given that the DBA/2J chromosome 2 segment contains a null mutation in the Complement Component 5 (C5) gene and other published studies implicating complement proteins in regulating Aβ deposition, it was hypothesized that deletion of C5 was responsible for the reduced Aβ deposition in the congenic strain. To examine this in greater detail, animals deficient for the C5a receptor (C5aR), one of the major pathways through which C5 signals, were mated to two different AD mouse models revealing a reduction in Aβ deposition. Taken
together, these studies suggest that C5/C5aR represents a unique signaling pathway that regulates Aβ deposition.

INTRODUCTION

Alzheimer’s disease (AD) is a progressive form of dementia associated with several pathological hallmarks including extracellular aggregates of Aβ peptide, senile plaque. The amyloid hypothesis of Alzheimer's disease (AD) suggests that altered Aβ metabolism is central to the pathogenesis of AD (Hardy and Selkoe, 2002). Therefore, identification of genetic pathways that can alter Aβ levels becomes important for creation of therapies that can slow the progression or delay the onset of the disease. While initially very effective in identifying genes associated with regulation of Aβ levels and AD, mostly autosomal dominant factors, human association studies have proven largely ineffective in reproducibly identifying genetic risk factors for LOFAD.

In an attempt to identify genetic factors that modulated Aβ deposition, this set of experiments utilized a unique mouse model of AD, the R1.40 congenic. This model contains the entire human APP gene, including its endogenous promoter and a disease causing mutation. It has been shown to recapitulate APP processing, Aβ metabolism and deposition seen in human AD (Kulnane and Lamb, 2001; Lamb et al., 1999; Lamb et al., 1997; Lehman et al., 2003b). The R1.40 transgene has been backcrossed onto the C57BL/6J (B6-R1.40) and DBA/2J (D2-R1.40), inbred strains for over 10 generations, facilitating identical insertion of the transgene onto both genetic backgrounds (Lehman et al., 2003a).
Therefore, variation in disease phenotype in each line maybe attributable to loci within the genetic background strain. Initial screening of these lines revealed the B6-R1.40 had high levels of steady state Aβ early in life corresponding to Aβ deposition late in life. In contrast, the D2-R1.40 line had low levels of Aβ early in life and did not deposit until very late in life. Interestingly, both strains had comparable levels of full length APP, the precursor to Aβ, as well as proteolytic processing products, CTFβ, indicative of formation of Aβ (Lehman et al., 2003a).

Subsequent F2 qualitative trait locus mapping (QTL) between the B6-R1.40 and D2-R1.40 lines, looking for an association between reduced steady state brain Aβ levels early in life and genetic loci within the D2 strain, revealed there were several genes responsible for the reduction in Aβ levels seen in the D2-R1.40 animals and in particular that there was a highly significant association between the reduced Aβ levels and a region on mouse chromosome 2 (Ryman et al., 2008). Analysis of mouse chromosome 2 revealed nine genes with non-synonymous coding polymorphisms: complement component 5 (C5, in the mouse also called Hc); zinc finger and btb containing domain 6 (Zbtb6); nuclear receptor subfamily, group A, member 1 (Nr5a1); low-density lipoprotein-related protein 1b (Lrp1b); zinc finger homeobox 1b (Zfhx1b); N-myc (and STAT) interactor (Nmi), Rap1 interacting factor 1 homolog (Rif1); nebulin (Neb); ADP-ribosylation factor-like 6 intereact protein 6 (Arl6ip6). Notably, the C5 gene has a loss of function mutation resulting in a complete absence of the C5 protein in the DBA2/J genetic background (Nilsson and Muller-Eberhard, 1967; Ooi and Colten, 1979; Wetsel et al., 1990).
C5 is an integral component of the complement system, part of the body’s innate immune response. The complement cascade can be activated by Aβ, resulting in cleavage of C5 into a powerful anaphylatoxin, C5a, which recruits microglia and astrocytes by binding to C5a receptors (C5aR) on the surface of these cells (Bradt et al., 1998).

The current studies examined the role of C5/C5aR signaling in modulating Aβ deposition. First, a congenic strain was created in which the C57BL/6J chromosome 2 locus implicated in the QTL studies was replaced by that from DBA/2J. Mating of this strain to the B6-R1.40 mouse model of AD, revealed that Aβ deposition was reduced relative to the B6-R1.40 controls. Second, to further examine the potential role of C5aR in modulating Aβ deposition animals deficient in the receptor were mated to two different mouse models of AD. Notably, in both transgenic models, C5aR deficiency lead to reduced Aβ deposition, suggesting that C5 signalling through C5a may promote Aβ deposition through mechanisms that have yet to be established but could involve altered microglial or astroglial removal of Aβ.

**MATERIALS AND METHODS**

**Animals**

The B6.D2.2D congenic animals were created by an initial backcross of the DBA2/J animals deficient for the C5 gene to the C57BL/6J strain. Offspring were genotyped and animals deficient for C5 were successively backcrossed to animals on the C57BL/6J strain. The C5a receptor knockout mouse
[C5ar1^tm/Cge/J (C5arKO)] was kindly provided by Craig Gerard (Harvard, Boston, MA). This transgenic line was created by using the phosphoglycerate kinase promoter driven by the neomycin resistance gene to replace the entire coding region of the endogenous C5aR gene. The construct was injected into 129S4/svJae mice and backcrossed to C57BL/6J (Hopken et al., 1996). The R1.40 transgene is a complete, full-length copy of the human APP gene (the 290 kb human APP gene carried on a 650 kb yeast artificial chromosome) containing the human early onset familial AD mutation K670N/M671L. Creation of the R1.40 transgenic mouse line and subsequent backcrossing to the C57BL/6J and DBA2/J (The Jackson Laboratory, stock number 000664 and stock number 000671 respectively) strains was described previously (Kulnane and Lamb, 2001; Lamb et al., 1999; Lamb et al., 1997; Lehman et al., 2003b). The APPPS1 transgenic line was kindly provided by Mathias Jucker (University of Tuebingen, Tuebingen, Germany). This line was generated by coinjection of the Thy1-APPKM670/671NL and Thy1-PS1L166P constructs into a pure C57BL/6J genetic background (Radde et al., 2006).

The R1.40 transgene was incorporated into the B6.D2.2D congenic animals as follows. Nontransgenic B6.D2.2D congenic animals homozygous for the D2 congenic interval were crossed to homozygous B6-R1.40 animals. The resulting animals that were hemizygous for the R1.40 transgene and heterozygous for the D2 congenic interval were subsequently intercrossed to generate animals that were hemizygous for the R1.40 transgene and homozygous for the congenic interval (for analysis of APP processing and Aβ
metabolism) and animal that were homozygous for the R1.40 transgene and the congenic interval (for analysis of Aβ deposition). Unfortunately, the APPPS1 transgene is located on mouse chromosome 2 in close proximity to the C5 locus (M. Jucker, personal communication), making generation of the B6.D2.2D-APPPS1 animals impossible.

Homozygous C5aRKO animals were crossed to both homozygous B6-R1.40 animals as well as hemizygous APPPS1 animals. Animals that were heterozygous for C5aRKO and hemizygous for the R1.40 transgene were intercrossed to generate homozygous C5aRKO mice that were either hemizygous (for analysis of APP processing and Aβ metabolism) or homozygous (for analysis of Aβ deposition) for the R1.40 transgene. In contrast, animals that were heterozygous for C5aRKO and hemizygous for the APPPS1 transgene were mated to hemizygous C5aRKO mice to generate mice that were homozygous C5aRKO and hemizygous for the transgene.

Animals in all experiments were age- and gender-matched. Animals were housed at the Cleveland Clinic Biological Resource Unit in ventilated cages. Temperature was maintained at 22°C and the light cycle was 14:10 h light:dark. Food and water were provided ad libitum.

**Tissue Procurement**

At 28 days, 3 male and 3 female hemizygous B6.D2.2D-R1.40, C5aRKO-R1.40, B6-R1.40, and D2-R14.0 animals were sacrificed by cervical dislocation. The brains were hemisected along the midline and snap frozen on dry ice. The
right and left hemispheres were stored at -80°C for western blot analysis of holo-
APP levels and APP processing and ELISA analysis of steady state Aβ1-40 levels respectively. For analysis of brain pathology, 3 male and 3 female four month old C5aRKO-APPPS1 and B6-APPPS1 mice and 3 male and 3 female 24 month old B6.D2.2D-R1.40, C5aRKO-R1.40, D2-R1.40 and B6-R1.40 animals were sacrificed by cervical dislocation and hemisected along the midline. One hemisphere was drop fixed in 10% formalin and embedded in paraffin.

**Western Blot Analysis of APP Processing**

The left hemisphere of 28 day old animals was analyzed for steady state levels of APP processing products, including holo-APP, CTFβ and CTFα. Frozen tissue was weighed and placed on dry ice until diluted with homogenization buffer. Tissue was homogenized in 24 volumes of 1% CHAPs in PBS using the Powergen 129 homogenizer (Prasher et al., 1998). Homogenates were mixed at 4°C for 30 minutes, then spun at 17,000 × g for 90 min. Protein concentration was determined using the BCA protein assay kit (Therma Fisher Scientific). Equal amounts of total protein (50 µg) was combined with LDS and reducing agent and heated at 70°C for 10 min. Samples were run on 4-12% Bis-Tris NuPAGE gels (Invitrogen), proteins transferred to PVDF membrane (Millipore) followed by blocking in 5% non-fat milk. Primary antibodies include 369 (1:2000, kindly provided by S. Gandy) which recognizes both holo-APP and and APP CTFs; glyceraldehydes 3-phosphate dehydrogenase (GAPDH) (1:40,000, Jackson ImmunoResearch Laboratories, catalog #115-035-003) as a loading
control were incubated with the blot in 5% nonfat milk and TBS-0.1% Tween for 1 hour at room temperature. Following washes, secondary antibodies (Licor), including anti-rabbit conjugated to IR 700 (1:20,000) and anti-mouse conjugated to IR 800 (1:20,000) were incubated with the blot in 5% nonfat milk, TBS-0.1% Tween, 0.01% SDS for 1 hr at room temperature. The membrane was washed 5 x 5 min in 1 x PBS and scanned wet on Odyssey Imaging System (LI-COR Biosciences) to visualize and quantify bands. Samples were normalized to the GAPDH signal as an internal loading control.

**Brain Aβ<sub>1-40</sub> ELISA Analysis of Steady State Aβ Levels**

Steady state brain Aβ levels were determined utilizing a human-specific fluorescent Aβ<sub>1-40</sub> ELISA (Biosource International) according to the manufacturer’s instructions. Briefly, the left hemisphere was homogenized in 5 M guanidine–100 mM Tris, pH 8.0 (1:8) and incubated at room temperature for 4 h with constant agitation to ensure complete protein extraction. Samples were diluted (1:20) in standard/sample buffer and centrifuged at 16,000 x g for 20 min at 4°C before being loaded in triplicate onto the ELISA plate for subsequent incubation with antibodies and fluorescent detection using known concentrations of Aβ peptides to create a standard curve.

**Immunohistochemical Analysis of Aβ Deposition**

Paraffin embedded brains from 4 month old C5aRKO-APPSS1 and B6-APPSS1 animals were cut into 10 µm sections and stained with Thioflavin S.
Briefly, slides were rehydrated by incubation in xylene, 100% EtOH, 95% EtOH, 70% EtOH, and H$_2$O, followed by incubation in a 1% solution of the amyloid-specific dye, Thioflavin S. Coverslips were mounted using Vecta-Shield Hard Set containing a DAPI counterstain (Vector Laboratories). Slides were examined under the fluorescent microscope.

10 µm paraffin sections from 24 month old B6.D2.2D-R1.40, C5aRKO-R1.40, D2-R1.40 and B6-R1.40 animals were stained for Aβ deposition using 6E10 (Senetek, Napa, CA), a mouse monoclonal antibody directed against Aβ1-17, as described previously (Kulnane and Lamb, 2001).

Amyloid deposition was assessed in the primary somatosensory cortex for the C5aRKO-APP-PS1 and B6-APP-PS1 animals and the entire cortex in the B6.D2.2D-R1.40, C5aRKO-R1.40, D2-R1.40 and B6-R1.40 animals. For quantitative analysis of Aβ deposition, two sections from each animal, the first medial, the other 250 µm lateral to the first were analyze with Image J (http://rsbweb.nih.gov/ij/). The sections spanned mouse brain Bregma locations from lateral 0.475mm to lateral 2.15mm (Allen Mouse Brain Atlas, http://mouse.brain-map.org/atlas/ARA/Sagittal/browser.html). Plaque counts were determined following specific criteria, edges were excluded and threshold was adjusted for size and circularity. The analyses were performed blinded to genotype.
Mapping of Congenic Interval

Genomic DNA was extracted from tail snips of B6.D2.2D-R1.40, B6-R1.40 and D2.R1.40 animals using TNES (50 mM Tris buffer, 0.5% SDS, 20 mM EDTA, 400 nM NaCl) and proteinase K digestion, followed by NaCl/Ethanol precipitation (Donnan, 2001). The DNA pellet was resuspended in a 10 mM Tris-HCL/1 mM EDTA solution. DNA concentration was determined using the Picogreen fluorescence assay (Invitrogen). The concentration of all DNAs was adjusted to a concentration of 50-150 ng/µL. The samples were genotyped using a Mouse Medium Density SNP Genotyping Array (Illumina) containing 1,449 SNP with a density of approximately 3 SNPs per 5 Mb intervals across the genome with at least 1 SNP per interval chosen to be informative for crosses involving the C57BL/6J strain by the Genomics Core in the Lerner Research Institute at the Cleveland Clinic.

Statistical Analysis

Statistical analysis of the levels of holo-APP, CTFs and Aβ was determined by one-way analysis of variance (ANOVA) using PRISM (GraphPad). The differences in Aβ deposition was assessed using a t-test in PRISM (GraphPad).
RESULTS

Generation of a B6.D2.2D Congenic Strain

The B6.D2.2D congenic animals were generated by successive backcrossing of an animal naturally deficient for C5, DBA/2J, to the C57BL/6J strain each time selecting for an animal that has a DBA/2J copy of C5. After 10 generations, the B6.D2.2D line was crossed to the B6-R1.40 animals and mated to homozygosity for both the congenic interval and the transgene.

Analysis of DNA from B6-R1.40, D2-R1.40 and B6.D2.2D-R1.40 animals identified a congenic interval approximately 25 Mb in size (Figure 3-1). It spans from marker CEL-2_32981944 (32,954,115 bp, Feb 2006 Assembly) to marker rs3672729 (58,115,109 bp, Feb 2006 Assembly). This correlates to the region of mouse chromosome 2 found to be associated with reduced Aβ levels in the QTL analysis [suggestive association starting at CEL-2_32981944 (32,954,115 bp, Feb 2006 Assembly) that becomes significant at SNP rs13476439 (38,043,281 bp, Feb 2006 Assembly) through SNP rs3718711 (56,461,355 bp, Feb 2006 Assembly)] (Figure 3-1) (Ryman et al., 2008).

APP Processing Unaltered in the B6.D2.2D-R1.40

As previous studies had suggested that the levels of APP CTFs were under genetic control in the B6-R1.40 and D2-R1.40 strains, the levels of holo-APP and APP CTFs in the B6.D2.2D-R1.40 strain were examined. Western blot analysis of brain homogenates from B6-R1.40, D2-R1.40 and B6.D2.2D-R1.40 animals revealed that there were no significant differences in either the levels of
holo-APP or the APP processing products, CTF\textsubscript{α} and CTF\textsubscript{β} (Figure 3-2). This suggests that the mechanisms responsible for alterations in steady state A\textsubscript{β} levels may be independent of APP processing.

**A\textsubscript{β} Levels Unaltered by the B6.D2 Congenic Interval of Mouse**

**Chromosome 2**

To examine the effects of the B6.D2.2D congenic interval on A\textsubscript{β} metabolism, hemizygous R1.40 mice containing the chromosome 2 locus from DBA/2J were generated by mating homozygous R1.40 mice to the B6.D2.2D congenic strain and animals analyzed at 28 days of age, as previously described. Notably, there were no significant differences in the steady state levels of A\textsubscript{β} between the B6-R1.40 and B6.D2.2D-R1.40 mice (Figure 3-3). However, as the QTL analysis suggested that the chromosome 2 locus contributed only 4\% of the variance in the levels of A\textsubscript{β} between the D2-R1.40 and B6-R1.40 strain, it remained possible that a significant difference would only be detected in an extremely large number of animals. Based on the number of samples and their standard deviation, the 0.1 mean difference between B6-R1.40 and B6.D2.2D-R1.40 animals has only 10\% power. Power analysis predicts at least 200 animals per group are required to say the difference is significant.
The B6.D2 Congenic Interval of Mouse Chromosome 2 Reduces Aβ Deposition

B6-R1.40, B6.D2.2D-R1.40 and D2-R1.40 animals were aged to 24 months of age to examine for the effects of the D2 chromosome 2 locus on Aβ deposition. Paraffin embedded brain sections were stained with monoclonal antibody 6E10, which recognizes human Aβ. While not statistically significantly different, quantification of brain sections revealed that the B6.D2.2D-R1.40 animals had a level of Aβ deposition that was lower than the B6-R1.40 animals, but higher than the D2-R1.40 animals (p=0.48 by one-way ANOVA with Tukey’s multiple comparison test, Figure 3-4). Taken together, this data supports the QTL studies and suggests that a locus within the D2 chromosome 2 congenic interval is responsible for the reduction of Aβ deposition.

Candidate Genes in the B6.D2.2D Congenic Interval

The B6.D2.2D congenic interval has synteny to human chromosome 9q33 and human chromosome 2q22-24. Notably, genetic association studies in human AD have identified significant AD loci on human chromosome 9q31, 9q34, 2q22, 2q24 (Lee et al., 2004; Myers et al., 2002; Pericak-Vance et al., 2000). The B6.D2.2D congenic interval contains about 100 genes (UCSC Genome Browser, genome.ucsc.edu). Nine of these genes contain nonsynonymous coding polymorphisms between the C57BL/6J and DBA/2J strains (Ryman et al., 2008). Two of these genes, complement component 5 (C5, 34805340 bp - 34883405 bp Feb 2006 Assembly) and low density lipoprotein receptor 1b
(Lrp1b, 40418782 bp – 42475607 bp Feb 2006 Assembly) have been previously associated with alterations in Aβ deposition and Aβ levels respectively (Cam et al., 2004; Fonseca et al., 2009). A third gene prostaglandin-endoperoxide synthase 1 (Ptgs1 or Cox1, 36052536 bp – 36074271 bp Feb 2006 Assembly) has been associated with AD in the literature, but contains only intronic polymorphisms (SNP Data in MGI, www.informatics.jax.org/strains_SNPs.shtml) (Hoozemans et al., 2008).

**Complement Component 5**

Complement component 5 (C5) was subsequently selected as a candidate gene for several reasons. First, in 35% of inbred strains, including the DBA2/J strain, C5 gene has a naturally occurring 2 bp deletion resulting in a premature stop codon and an absence of C5 protein and its downstream cleavage products C5a and C5b (Nilsson and Muller-Eberhard, 1967; Ooi and Colten, 1979; Wetsel et al., 1990). Second, absence of C5 has been shown to increase susceptibility of neurons to death (Pasinetti et al., 1996). Third, complement has been associated with AD including: activation of the complement cascade by Aβ, the presence of complement proteins in Aβ deposits in the human AD brain as well as and experimental evidence that the absence of complement proteins modulates Aβ levels (Fonseca et al., 2004; Matsuoka et al., 2001; Rogers et al., 1992; Wyss-Coray et al., 2002; Yang et al., 2000). The presence of a loss of function allele in C5 in the B6.D2.2D congenic strain led us to hypothesize that loss of C5 signaling could be responsible for the observed reduction in Aβ
deposition in the B6.D2.2D-R1.40 strain. C5 is proteolytically cleaved into C5a and C5b. While it is unclear if C5b goes on to form the membrane attack complex (MAC) in the brain, experimental evidence supports the role of C5a in Aβ clearance through C5a-C5aR signaling (Fonseca et al., 2009; Woodruff et al., 2010).

**Altered APP Processing is Not Responsible for Changes in Aβ Deposition**

To examine the effects of C5aR deficiency on holo-APP levels and APP processing, brains from 28 day old mice were analyzed via Western blot using polyclonal antibody 369, which recognizes both holo-APP as well as both APP CTFs. An antibody against GAPDH was utilized as an internal loading control. Quantification and analysis of the levels of holo-APP, CTFα and CTFβ revealed no significant differences (Figure 3-5).

**Effects of C5aR Deficiency on Steady State Aβ Levels**

Steady state Aβ levels were examined at 28 days in the B6-R1.40, D2-R1.40 and C5aRKO-R1.40 animals using an Aβ1-40 ELISA. Aβ levels in the C5aRKO-R1.40 animals were not different from the B6-R1.40 animals (Figure 3-6). Again, this is because the difference in Aβ levels between the two strains is so small it would only be significant in a very large number of samples. Based on the number of animals per group and the standard deviation within each group, the 0.37 difference in mean between the B6-R1.40 and C5aRKO-R1.40 animals
has only 20% power. Power analysis states it would require 30 animals per
group to say if the difference in mean is significant.

**Absence of the C5a Receptor Reduces Aβ Deposition in 2 Mouse Models of AD**

To examine the role of C5 function through the C5aR on Aβ deposition, C5aR knockout mice in an inbred C57BL/6J background were obtained and mated with two mouse models of AD, B6-R1.40 and B6-APPPS1.

The first set of experiments, examined the effect of C5aR deficiency on Aβ deposition in the R1.40 mouse model. B6-R1.40, D2-R1.40 and C5aRKO-R1.40 animals were aged to 24 months of age. Analysis of paraffin embedded brain sections via immunohistochemistry with Aβ antibody 6E10 revealed deposition similar to D2-R1.40, but significantly less than B6-R1.40 mice (**Figure 3-7**).

To examine whether C5aR deficiency had a similar effect on Aβ deposition in a different mouse model of AD, the B6-APPPS1 mouse model of AD was utilized. Notably, this strain contains both the Swedish FAD mutation in the APP gene as well as an aggressive PSEN1 FAD mutation that results in extensive fibrillar Aβ deposition as early as two months of age. C5aR deficient B6-APPPS1 mice and B6-APPPS1 controls were aged to 4 months of age and examined for Aβ deposition by staining with Thioflavin S. Aβ deposition was significantly decreased in the C5aRKO-APPPS1 animals compared to the B6-APPPS1 animals (**Figure 3-8**).
DISCUSSION

Numerous epidemiological studies have demonstrated that AD is a complex, heterogeneous disorder with genetic factors accounting for ~80% of disease risk (Gatz et al., 2006). However, more than half of these genetic risk factors have yet to be identified due to small effect size, genotypic and phenotypic heterogeneity and environmental factors.

Genetic screens using mouse models of AD have been able to overcome these obstacles and identify associations with chromosomal loci involved in Aβ metabolism. One previous QTL study in the mouse between the B6-R1.40 and D2-R1.40 animals revealed a significant association with reduced Aβ and mouse chromosome 2 (Ryman et al., 2008). Congenic animals, animals completely C57BL/6J except for a segment of chromosome 2 corresponding to the QTL that was DBA/2J, were created and mated to the R1.40 mouse model of AD. Analysis revealed a reduction in Aβ deposition in the B6.D2.2D-R1.40 animals, confirming the findings of the QTL study.

Examination of the congenic interval identified one likely candidate gene responsible for alterations in Aβ. The C5 gene has a loss of function mutation in the DBA/2J strain and an absence of C5 and C5a, its downstream cleavage product that binds to the C5a receptor (C5aR). To examine the potential contribution of the C5 signaling pathway in altering Aβ deposition, the R1.40 mouse model of AD was mated to a C57BL/6J mouse model deficient for the C5a receptor. Absence of the C5aR reduced Aβ deposition. This was verified in
a second mouse model of AD, APPPS1, which also showed reduced Aβ deposition in the absence of C5aR. Taken together these results support a role of the C5 pathway regulating/modulating Aβ deposition.

Experimental evidence supports a role of complement in the pathogenesis of AD. Complement factors are associated with fibrillar Aβ deposits early in the disease (Aikayama et al., 2000; Loeffler et al., 2008; Zanjani et al., 2005). Several studies have demonstrated that Aβ can activate the complement pathway resulting in the downstream production of C5a and C3a, both of which are chemotactic for microglia and astrocytes (Afagh et al., 1996; Bradt et al., 1998; Matsouko et al., 2001; Rogers et al., 1992).

Previous studies in mouse models of AD have provided mixed findings with regard to the role of complement in AD. Two studies have shown that deficiency of anaphylatoxin C3, a component of the complement cascade upstream from C5, results in increased Aβ deposition and neurodegeneration potentially mediated via altered Aβ clearance in microglia (Maier et al., 2008; Wyss-Coray et al., 2002). However, absence of the recognition component of the classical complement cascade, C1q, did not alter Aβ deposition but instead resulted in altered gliosis and neurodegeneration (Fonseca et al., 2004). Finally, exposure of a mouse model of AD to a synthetic C5aR antagonist (PMX205) resulted in reduced Aβ deposition and microglial activation, resulting in neuroprotection (Fonseca et al., 2009). Variation in the effects of different components of the complement pathway on Aβ deposition is likely due to differences in the unique function of each complement factor examined. Indeed,
a recent study revealed that in the absence of C5-C5aR signaling, functions of C1q and C3 remain intact (Ager et al., 2010).

The current study supports the findings of the C5aR antagonist, that absence of C5a signaling results in reduced Aβ deposition. However, preliminary findings suggest no difference in microglial number or activation status in the B6.D2.2D-R1.40, C5aRKO-R1.40 or C5aRKO-APPSS1 mice compared to the B6-R1.40 or B6-APPSS1 (respectively) controls (data not shown).

There are several possible mechanisms by which Aβ deposition may be altered in the B6.D2.2D and C5aR animals. C5a is involved in functions essential to the clearance of pathogens for host defense, including increased vascular permeability, chemotaxis of inflammatory cells, cytokine and chemokine release and phagocytosis (Guo and Ward, 2005). Anaphylatoxin C5a is a proinflammatory chemoattractant molecule released locally in tissues at the site of complement activation (Woodruff et al., 2010). It functions through two receptors, the C5a receptor (C5aR) which is a classical G protein-coupled receptor and C5a receptor-like 2 (C5L2) which is homologous in structure to C5aR except for the G protein coupling (Bamberg et al., 2010). C5a binds to both receptors with similar affinity (Monk et al., 2007). The receptors for C5a are expressed on neutrophils, eosinophils, monocytes, macrophages, leukocytes, hepatocytes, epithelial cells, endothelial cells, tissue mast cells, astrocytes and microglia (Gasque et al., 1997). Studies have shown that C5aR can mediate toll-like receptor (TLR) signaling (Zhang et al., 2007). Therefore, inflammation in the presence of C5a may be enhanced and the absence of C5a may
correspondingly reduce gliosis. Notably, Aβ deposition induces an upregulation of C5aR within microglia surrounding plaques and the microglial processes closest to the plaque and this could lead to reduced Aβ clearance by microglia (Ager et al., 2010; Lee and Landreth, 2010). Removal of C5aR would correspondingly enhance the phagocytic removal of Aβ. Further studies are required to determine the mechanism by which Aβ is reduced as well as determine the effects on cognition.

The current study provides evidence that C5 is involved in the reduced Aβ deposition observed in the DBA/2J strain. These observations support the previous findings that inhibition of the C5a signaling pathway can alter Aβ deposition (Fonseca et al., 2009). Further studies are required to determine if this model also mimics the neuroprotection observed in the PMX205 inhibited animals as this model provides an attractive therapeutic treatment due to its specificity.
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Figure 3-1: Region identified in QTL mapping correlates with B6.D2.2D congenic interval. DNA extracted from mouse tail snips was analyzed by Illumina mouse medium density SNP genotyping array to determine the size of the DBA/2J locus introgressed into the C57BL/6J chromosome 2 in the B6.D2.2D strain. The boundaries for the congenic interval CEL_32981944 (32954115 bp) and rs3672719 (58115109 bp), black. The region is ~25Mb and contains ~100 genes. QTL analysis revealed a significant association with reduced Aβ levels and the region of mouse chromosome 2 with boundaries CEL_32981944 (32954115 bp) and rs371711 (56461355 bp), with maximum linkage at 41216315 bp, grey.
**Figure 3-2:** B6.D2.2D congenic interval does not alter APP processing. B6-R1.40, D2-R1.40 and B6.D2.2D-R1.40 were sacrificed at 28 days of age. CHAPs brain extracts were analyzed for holo-APP and CTF levels by Western blot. There was no significant difference in holo-APP or $\text{CTF}_\beta$ between the 3 strains as determined by a one-way ANOVA with Tukey’s multiple comparison test.
B6 - B6 - D2 - D2

pmol/g brain

B6-R1.40  B6.D2.2D-R1.40  D2-R1.40

p<0.01 *
Figure 3-3: B6.D2.2D congenic interval on chromosome 2 does not alter steady state $A\beta$ levels. B6-R1.40, D2-R1.40 and B6.D2.2D-R.140 animals were sacrificed at 28 days of age. Guanidine brain extracts were analyzed for steady state levels of $A\beta_{1-40}$ by ELISA. There was no difference between B6-R1.40 and B6.D2.2D animals, but both were significantly different than the D2-R1.40 animals ($p<0.01$) as determined by one-way ANOVA with Tukey’s multiple comparison test.
B6-R1.40

B6.D2.2D-R1.40

D2-R1.40

![Images of brain sections with amyloid burden analysis](chart.png)

**Amyloid Burden**

- **B6-R1.40**: 0.0025 ± 0.0005
- **B6.D2.2D-R1.40**: 0.0015 ± 0.0005
- **D2-R1.40**: 0.0010 ± 0.0005
Figure 3-4: D2 congenic interval on mouse chromosome 2 reduces Aβ deposition. B6-R1.40, D2-R1.40 and B6.D2.2D-R1.40 were sacrificed at 24 months of age. The cortex of paraffin fixed sections were analyzed for Aβ deposition using 6E10 antibody (2 sections/animal) and Image J to identify plaque area. While not statistically significantly different, the B6.D2.2D-R1.40 has an intermediate level of Aβ deposition compared to the B6-R1.40 and D2-R1.40 animals (p=0.48 by one way ANOVA with Tukey’s multiple comparison test).
Figure 3-5: Absence of the C5a receptor does not alter APP processing.

B6-R1.40, D2-R1.40 and C5aRKO-R1.40 animals were sacrificed at 28 days of age. CHAPs brain extracts were analyzed for holo-APP and CTF levels by Western blot. While the level of APP in C5aRKO-R1.40 animals appears to be increased, there is no statistical difference. Likewise, there is no difference in the level of CTFβ between the three strains as determined by one-way ANOVA with Tukey’s multiple comparison test.
Figure 3-6: Absence of C5a receptor does not alter steady state Aβ levels. B6-R1.40, C5aRKO-R1.40 and D2-R1.40 animals were sacrificed at 28 days of age. Guanidine brain extracts were analyzed for steady state levels of Aβ1-40 by ELISA. The D2-R1.40 animals have significantly less Aβ than the B6-R1.40 and C5aRKO-R1.40 animals (p<0.01) as determined by a one-way ANOVA with Tukey’s multiple comparison test.
**Figure 3-7: Absence of the C5a receptor reduces Aβ deposition in the R1.40 mouse model of AD.** B6-R1.40, C5aRKO-R1.40 and D2-R1.40 animals were sacrificed at 24 months of age. The cortex of paraffin embedded sections were analyzed for Aβ deposition using 6E10 antibody (2 sections/animal) and Image J to identify plaque area. While not statistically significantly different, the C5aRKO-R1.40 animals have reduced Aβ deposition compared to the B6-R1.40 animals, similar to the deposition seen in the D2-R1.40 animals (p=0.32 by one way ANOVA with Tukey’s multiple comparison test).
Figure 3-8: Absence of C5a receptor reduces Aβ deposition in the APPPS1 mouse model of AD. C5aRKO-APPPS1 and B6-APPPS1 animals were sacrificed at 4 months of age. The somatosensory cortex of paraffin embedded sections were analyzed for Aβ deposition using Thioflavin S (2 sections/animal) and Image J. There was a significant decrease in the area occupied by Aβ deposits in the C5aRKO-APPPS1 animals (p=0.015) as determined by t-test.
Chapter 4: Genetic Background Differentially Influences Beta-Amyloid Metabolism and Deposition

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ABSTRACT

Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by distinct neuropathological hallmarks including deposits of β-amyloid (Aβ) peptide within the brain parenchyma. Human genetic studies have provided strong evidence that early-onset familial AD share common pathogenic mechanisms that involve relatively subtle alterations in the amyloid precursor protein (APP) and Aβ metabolism. To identify genetic factors that modify Aβ metabolism and deposition within the brain, our laboratory has undertaken a genetic screen using the mouse model system. Studies have demonstrated that introduction of the genomic-based R1.40 transgene into different inbred mouse strains, resulted in genetic based alterations in Aβ metabolism. The current study further characterizes the effect of genetic background in modifying Aβ metabolism and deposition in the R1.40 mouse model of AD. In particular, these studies demonstrate that the R1.40 transgene in the C57BL/6J background results in elevated Aβ levels and age-related Aβ deposition. By contrast, the R1.40 transgene in the 129S1/SvImJ and DBA/2J genetic background results in reduced Aβ levels and dramatically reduced Aβ deposition. Notably, the R1.40 transgene in the A/J background results in Aβ levels even higher than that observed in C57BL/6J mice and yet exhibit no Aβ deposition later in life. Finally, using chromosome substitution and congenic strains, genetic factors that regulate Aβ deposition in the mouse are being identified. In particular, a locus on A/J chromosome 11 as well as a separate locus on DBA/2J chromosome 1 has been identified that reduces Aβ deposition in a transgenic mouse model of AD.
INTRODUCTION

Alzheimer’s disease (AD) is a progressive form of dementia marked by several pathological hallmarks including extracellular deposits of the 40-42 amino acid peptide, beta-amyloid (Aβ). A substantial body of experimental evidence, including both genetic, biochemical and pathological studies suggest that the generation and deposition of the Aβ peptide is central to the disease process and has lead to the amyloid hypothesis of AD (Hardy and Selkoe, 2002; Selkoe, 2008). The amyloid hypothesis stipulates that it is the relative amounts of specific Aβ isoforms, oligomers and/or fibrils that drives AD pathogenesis. Notably, mutations in the amyloid precursor protein (APP), the immediate precursor to Aβ as well as the presenilins, the catalytic subunit of the enzyme that generates Aβ from APP are both associated with early-onset familial AD. Similarly, duplication of the APP gene is also associated with autosomal dominant AD.

Twin studies have provided evidence that genetic factors greatly influence AD risk, with estimates of 40-80% of the disease accounted for by genetic factors and the remainder of the risk attributed to poorly defined environmental and stochastic factors (Gatz et al., 2006). Thus far, it is estimated that only ~30% of the genes responsible for altered AD risk have been identified (Tanzi and Bertram, 2001). The remaining 70% have proven difficult to identify for a number of postulated reasons including the presence of numerous genes with small
effect sized necessitating the use of very large sample sizes in current case-control studies.

To identify additional genetic factors that modulate Aβ levels and Aβ deposition, several laboratories have turned to genetic mouse models of AD. For example, the R1.40 transgenic mouse model of AD, which contains the entire human APP gene with an early-onset familial AD mutation generates substantial levels of Aβ peptides and develops age-related Aβ deposits and other AD phenotypes.

Notably, generation of mice with the R1.40 transgene on different genetic backgrounds demonstrated unique patterns of APP processing, Aβ metabolism and Aβ deposition. For example, R1.40 animals in the C57BL/6J background (B6-R1.40) generate high levels of Aβ peptides within the brain, associated with an age-related accumulation of Aβ deposits. By contrast, R1.40 animals in the DBA/2J background (D2-R1.40), exhibit reduced levels of brain Aβ with minimal age-related deposition of Aβ. Finally, R1.40 animals in the 129S1/SvImJ strain generate intermediate levels of Aβ, but the effects on Aβ deposition have thus far not been assessed (Lehman et al., 2003a). In a separate unrelated study, a genetic screen was conducted using the TgCRND8 transgenic mouse model of AD, which identified genetic loci on A/J chromosomes 4, 9 and 11 that reduced Aβ deposition.

The current study extended the characterization of the R1.40 transgene on different genetic backgrounds and also identified unique chromosomal regions that influence Aβ deposition in the mouse. First, analysis of Aβ deposition in the
129S1-R1.40 strain revealed only minimal age-related deposition. Second, generation and analysis of R1.40 animals in the A/J genetic background (A-R1.40) revealed elevated steady state levels of brain Aβ compared to B6-R1.40 animals and yet no detectable Aβ deposition in aged animals. Finally, using genetic resources available in the mouse, unique chromosomal loci from the DBA/2J (on chromosome 1) and A/J (on chromosome 11) inbred strains were identified that reduce Aβ deposition in a transgenic mouse model of AD. Together, these studies demonstrate the different patterns of Aβ metabolism and deposition are influenced by unique genetic factors present in inbred mouse strains and tentatively identifies two genetic loci that regulate Aβ deposition.

MATERIALS AND METHODS

Animals

The R1.40 transgene is a complete, full-length copy of the human APP gene (the 290 kb APP gene carried on a 650 kb yeast artificial chromosome) containing the early onset familial AD mutation K670N/M671L. Creation of the R1.40 transgenic mouse strain and subsequent backcrossing to the C57BL/6J (The Jackson Laboratory stock number 000664), DBA/2J (stock number 000671), 129S1/SvImJ (stock number 002448) and A/J (stock number 000646) strains to generate the B6.129-Tg(APPsw)40Btla/J (B6-R1.40), D2.129(B6)-Tg(APPsw)40Btla/J (D2-R1.40), 129S1.129(B6)-Tg(APPsw)40Btla/J (129S1-R1.40), and A.129(B6)-Tg(APPsw)40Btla/J (A-R1.40) lines was described previously. (Lamb et al., 1997; Lamb et al., 1993; Lehman et al., 2003a) The B6-
APPoS1 mouse model of AD (kindly provided by Mathias Jucker, University of Tuebingen, Tuebingen, Germany) was generated by coinjection of the Thy1-APPKM670/671NL and Thy1-PS1L166P constructs into a pure C57BL/6J genetic background (Radde et al., 2006). Chromosome substitution strains (CSSs) in which A/J chromosomes 4, 9 and 11 were introduced into a pure C57BL/6J background were obtained from Jackson Laboratory (C57BL/6J-Chr4^A^/NaJ, C57BL/6J-Chr9^A^/NaJ and C57BL/6J-Chr11^A^/NaJ, respectively). The CSSs were crossed to both the R.140 transgene (B6.A4-R1.40, B6.A9-R1.40, B6.A11-R1.40) as well as the APPoS1 transgene (B6.A4-APPPS1, B6.A9-APPPS1, B6.A11-R1.40). C57BL/6J strains in which a specific segment of chromosome 1 (from markers rs6267646 to mCV24145570) and chromosome 7 (from markers rs3689765 to rs6322316) from the DBA/2J strain (B6.D2.1D and B6.D2.7D, respectively) were obtained from Richard Davis (Davis et al., 2005; Iakoubova et al., 2001).

B6.A consomic animals of all groups (B6.A4-R1.40, B6.A4-APPPS1, B6.A9-R1.40, B6.A9-APPPS1, B6.A11-R1.40, B6.A11-PS1APP) were generated as follows. Homozygous B6.A consomic animals were crossed to homozygous B6-R1.40 mice as well as hemizygous B6-APPPS1 mice. Animals hemizygous for the respective transgene and heterozygous for the A/J chromosome were identified and subsequently mated to the respective homozygous B6.A animals. Analysis of APP processing and Aβ metabolism were conducted on animals homozygous for the A/J chromosome and hemizygous for the R1.40 transgene. Animals homozygous for the A/J chromosome and hemizygous for the APPoS1...
transgene were used to analyze Aβ deposition. Homozygous B6.D2 congenic animals were crossed to homozygous B6-R1.40 mice as well as hemizygous B6-APPSS1 mice. Animals that were hemizygous for the transgene and heterozygous for the B6.D2 congenic interval were identified and subsequently mated to the respective homozygous B6.D2 animals. Analysis of APP processing and Aβ metabolism were conducted on animals that were homozygous for the B6.D2 congenic interval and hemizygous for the R1.40 transgene. Animals homozygous for the B6.D2 congenic interval and hemizygous for the APPSS1 transgene were used to analyze Aβ deposition.

Animals in all experiments were age- and gender-matched. Animals were housed at the Cleveland Clinic Biological Resource Unit, in ventilated cages. Temperature was maintained at 22°C with a 14:10 h light:dark cycle. Food and water were provided ad libitum.

**Tissue Procurement**

3 male and 3 female R1.40 transgenic animals were sacrificed at 28 days by cervical dislocation. The right and left hemispheres were snap frozen on dry ice and stored at -80°C for Western blot analysis of holo-APP and APP CTFs and ELISA of steady state levels of Aβ1-40. Likewise, 3 male and 3 female 24 month old R1.40 transgenic animals were sacrificed by cervical dislocation. The brains were hemisected along the midline, one half was snap frozen on dry ice and stored at -80°C for Western blot analysis of holo-APP and APP CTFs and ELISA of steady state levels of Aβ1-40. The other hemisphere was drop fixed in 10%
formalin and embedded in paraffin for immunohistochemical analysis of Aβ deposition.

3 male and 3 female 4 month old APPPS1 transgenic animals were perfused with 1x phosphate buffer (PB). The brains were removed and sagittally bisected. Brains were immersion fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline for immunohistochemical analysis of Aβ deposition. After 24 hours, brains were removed from PFA and cryoprotected with 30% sucrose in PB. Tissue was embedded with O.C.T. embedding medium (Miles, Inc) for cryosectioning.

**Brain Aβ1-40 ELISA - Analysis of Steady State Aβ Levels**

Steady state brain Aβ levels were determined utilizing a human-specific fluorescent Aβ1-40 ELISA (Biosource International) according to the manufacturer's instructions. Briefly, each hemisphere was homogenized in 5 M guanidine–100 mM Tris, pH 8.0 (1:8) and incubated at room temperature for 4 h with constant agitation to ensure complete protein extraction. Samples were subsequently diluted (1:20) in standard/sample buffer and centrifuged at 16,000 x g for 20 min at 4°C before being loaded in triplicate onto the ELISA plate for subsequent incubation with antibodies and fluorescent detection using known concentrations of Aβ peptides to create a standard curve.
Western Blot Analysis of APP Processing

The hemisphere of the brain was analyzed for steady state levels of APP processing products including holo-APP, CTF$_p$, and CTF$_\alpha$. Tissue was homogenized in 24 volumes of 1% CHAPs in PBS using the Powergen 129 homogenizer (Prasher et al., 1998). Homogenates were mixed at 4°C for 30 minutes, then spun at 17,000 x g for 90 min. Protein concentration was determined using the BCA protein assay kit (Therma Fisher Scientific). Equal amounts of total protein (50 µg) were combined with Lithium Dodecyl Sulfate (LDS) and reducing agent and heated at 70°C for 10 min. Samples were run on gradient 4-12% Bis-Tris NuPAGE gels (Invitrogen), proteins transferred to PVDF (Millipore), followed by blocking in 5% non-fat milk. Primary antibodies include polyclonal 369 (1:2000, kindly provided by S. Gandy) which recognizes both holo-APP and APP CTFs and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) (1:40,000, Jackson ImmunoResearch Laboratories, catalog #115-035-003) as a loading control were incubated with the blot in 5% non-fat milk and TBS-0.1% Tween for 1 hour at room temperature. Following washes, infrared secondary antibodies (Licor), including anti-rabbit antibody conjugated to IR 700 (1:20,000) and anti-mouse antibody conjugated to IR 800 (1:20,000) were incubated with the blot in 5% non-fat milk, 0.1% Tween, 0.01% SDS for 1 hour at room temperature. Western blot was subsequently visualized and quantified using the Odyssey Imaging System (LI-COR Biosciences) and all samples were normalized to the GAPDH signal as an internal loading control.
**Immunohistochemical Analysis of Aβ Deposition**

Paraffin embedded brains from the B6-R1.40, D2-R1.40, 129S1-R1.40 and A-R1.40 animals were cut into 10 μm sections and analyzed for Aβ deposition via immunohistochemistry utilizing the human-specific monoclonal Aβ antibody 6E10. Slides were heated on the slide warmer at 56°C for 30 minutes, followed by a 3 min wash in 70% formic acid. Slides were deparaffinized via incubation in xylene (2 x 5 min) followed by rehydration in decreasing concentrations of ethanol and finally water. Slides were subsequently incubated in 3% peroxide for 20 min followed a quick rinse in H₂O. The slides were microwaved in H₂O for 3 min, cooled, then rinsed prior to incubation with primary antibody 6E10 (1:1000) directed against Aβ1-17, as described previously (Kulnane and Lamb, 2001). Following washes, slides were incubated with secondary antibody mouse IgG (1:200), incubated in Avidin: Biotinylated enzyme Complex (Vectastain Elite ABC kit), incubated in DAB (3,3’-diaminobenzidine) solution (Sigma), counterstained with hematoxylin, and subsequently dehydrated through incubation in increasing concentrations of EtOH and mounted with coverslips.

For B6.A and B6.D2 studies, frozen, cryoprotected brains were cut into 10 μm sagittal sections. Sections were stained for Aβ deposition using Thioflavin S. Briefly, slides were rehydrated by incubation in xylene followed by decreasing concentrations of EtOH and incubation in a solution of 1% Thioflavin S. Slides were examined by fluorescent microscope. Cryosections from B6.A and B6.D2 animals were also stained for Aβ deposition using 6e10 antibody as described.
above. However, slides did not need to be deparaffinized and dilution of 6e10 primary antibody (1:500) was reduced. Images were captured using light microscope.

Amyloid deposition was assessed in the primary somatosensory cortex for the B6.A-APPSS1, B6.D2-APPSS1 and B6-APPSS1 animals and the entire cortex in the A-R1.40, 129S1-R1.40, D2-R1.40 and B6-R1.40 animals. For quantitative analysis of Aβ deposition, two sections from each animal, the first medial, the other 250 µm lateral to the first were analyze with Image J (http://rsbweb.nih.gov/ij/). The sections spanned mouse brain Bregma locations from lateral 0.475mm to lateral 2.15mm (Allen Mouse Brain Atlas, http://mouse.brain-map.org/atlas/ARA/Sagittal/browser.html). Plaque counts were determined following specific criteria, edges were excluded and threshold was adjusted for size and circularity. The analyses were performed blinded to genotype.

**Statistical Analysis**

Statistical analysis of the levels of holo-APP, CTFs and Aβ was determined by analysis of variance (ANOVA) using PRISM (GraphPad).

**RESULTS**

The congenic R1.40 mouse model was used to identify the presence of genetic modifiers of AD phenotypes. The R1.40 transgene on the C57BL/6J background exhibits a number of phenotypic features characteristic of AD
including elevated levels of steady state Aβ and Aβ deposition. To examine the effects of genetic background on influencing AD phenotypes, the R1.40 transgene was also introduced into the DBA/2J, 129S1/SvImJ and A/J genetic backgrounds. Our previous studies observed genetic based differences in APP processing, Aβ metabolism as well as Aβ deposition between the B6-R1.40 and D2-R1.40 congenic strains (Lehman et al., 2003a). Subsequent QTL analysis revealed genetic loci on DBA/2J mouse chromosomes 1, 2 and 7 that were associated with lower steady state levels of brain Aβ (Ryman et al., 2008).

**Further Characterization of R1.40 Congenic Strains**

As the initial studies on the R1.40 congenics focused on unique differences between the B6-R1.40 and D2-R1.40 animals, the current studies expanded this through an analysis and characterization of all four of the congenic strains. First, to determine the extent of the genomic region on mouse chromosome 13 inherited with the R1.40 transgene in each congenic strain, Illumina SNP genotyping was performed using the Medium Density SNP Genotyping Array, which has over 1,449 polymorphic SNPs with a density of approximately 3 SNPs per 5 Mb intervals across the genome. Notably, the size of the congenic interval was overall similar for the different strains. In addition, extensive Southern blot analysis revealed that the transgene exhibited a similar genomic organization and copy number (4-6) in all four congenic strains (data not shown).
**APP Processing**

Second, additional experiments were conducted to examine differences in APP processing and Aβ metabolism in the different congenic strains at different ages. Our initial experiments focused on an analysis at 28 days, as previous studies had demonstrated that the differences exhibited at 28 days were stable at later ages (up to 6 months of age). Brains from all four congenic strains were analyzed by Western blots for the steady state levels of APP and its processing products, CTFα and CTFβ (Figure 4-1A). Holo-APP levels between the B6-R1.40, D2-R1.40 and 129S1-R1.40 and A-R1.40 lines were identical. Likewise, the levels of CTFα as well as CTFβ were comparable in all four strains, B6-R1.40, D2-R1.40, 129S1-R1.40, A-R1.40. Furthermore, more detailed analysis of the APP CTFs and separation of all the different unphosphorylated and phosphorylated forms of APP CTFs revealed no significant differences between the four congenic strains (data not shown).

Next, to examine whether the levels of holo-APP and APP CTFs changed with age in the four congenic strains, animals at 6, 13, 17, 20 and 24 months of age were examined for the levels of brain holo-APP, CTFα and CTFβ via Western blot analysis. Similar to what was observed at 28 days, there were no significant differences in the levels of holo-APP, CTFα and CTFβ between the four congenic strains at any of the age groups examined. Furthermore, the levels of these APP products also did not exhibit significant differences within a congenic strain across the ages examined (data not shown).
**Steady State Aβ Levels**

The levels of steady state brain Aβ in the four congenic strains was determined by ELISA (Figure 4-1B). The studies focused on Aβ_{1-40}, as previous studies had demonstrated that both Aβ_{1-40} and Aβ_{1-42} exhibited a similar pattern in the congenic strains (data not shown). Notably, similar to what was published previously, the D2-R1.40 exhibit the lowest levels of brain Aβ, 129S1-R1.40 mice exhibit intermediate levels and B6-R1.40 animals exhibit elevated levels of Aβ. Surprisingly, the A-R1.40 strain exhibited the highest levels of steady state Aβ, that was significantly higher than all three of the other congenic strains.

**Aβ Deposition**

Finally, to examine the effect of genetic background on Aβ deposition, all four congenic strains were analyzed via Aβ immunohistochemistry of brain tissue (Figure 4-1C). Similar to previously published results, B6-R1.40 mice exhibited age-related Aβ deposition starting at 12-14 months, although there was considerable variation in the extent of Aβ deposition at a given age. Staining of brain sections from the D2-R1.40 and 129S1-R1.40 animals revealed very limited Aβ deposition, and only at advanced ages (>22 months), suggesting that Aβ deposition is substantially delayed in the D2-R1.40 and 129S1-R1.40 congenics relative to the B6-R1.40 congenic. Notably, analysis of the A-R1.40 cogenic strain revealed no detectable Aβ deposition at any of the ages examined, up to 28 months, in spite of the fact that the A-R1.40 strain exhibited the highest levels of steady state brain Aβ levels in young animals. Furthermore, steady state
levels of Aβ as measured by ELISA reflected the extent of Aβ deposition determined via immunohistochemistry, with an elevation in Aβ levels at ~13 months in the B6-R1.40 mice, at 20 months in the 129S1-R1.40 mice and 24 months in the D2-R1.40 mice (data not shown).

Taken together, the characterization of the R1.40 congenic strains demonstrated that there are unique genetic traits that influence Aβ metabolism and deposition in the mouse. In particular, these studies demonstrate that D2-R1.40 mice exhibit reduced brain Aβ levels and delayed Aβ deposition when compared to B6-R1.40 mice while A-R1.40 exhibit elevated steady state Aβ levels and yet do not develop Aβ deposits across the lifespan.

**Identification of A/J Genetic Loci that Alters Aβ Deposition**

Unique genetic resources available for this strain, termed chromosome substitution strains (CSSs) in which individual A/J chromosomes are present in an otherwise inbred C57BL/6J background were utilized to identify genetic loci that regulate Aβ deposition in the A/J mouse strain.

The choice of which A/J chromosomes to screen first for modifiers of Aβ deposition was dictated by previous findings in a separate transgenic mouse model of AD, TgCRND8. TgCRND8 is a cDNA-based transgenic mouse in which APP, with the APPK670N/M671L and APPV717F mutations, is driven by the PrP promoter (Chishti et al., 2001). Notably TgCRND8 mice maintained on a hybrid C57BL/6J X A/J background exhibited high variable Aβ deposition and a more detailed QTL analysis revealed prominent genetic loci on A/J chromosomes 4, 9
and 11 that reduced Aβ deposition in the TgCRND8 mice (Sebastiani et al., 2006).

Thus, the initial experiments focused on using the chromosome 4, 9 and 11 CSSs (termed B6.A4, B6.A9 and B6.A11) to identify genetic factors that regulate either Aβ metabolism or Aβ deposition. The current studies utilized two different mouse models of AD to examine the effects of A/J chromosomes on Aβ metabolism on the one hand and Aβ deposition on the other. The mating of the A/J CSSs to the B6-R1.40 mice provides important data regarding genetic loci that regulate the steady state levels of Aβ. On the other hand, the mating of the A/J CSSs to the B6-APPSS1 mice focused on the identification of specific A/J chromosomes that modify Aβ deposition. While it would have been ideal to utilize the genomic-based R1.40 mouse model AD for all of these studies, the fact that B6-R1.40 mice exhibit Aβ deposition that begins only at 12 months of age, suggests that these studies would be prohibitively costly and time-consuming. In an effort to more rapidly identify genetic factors that influence Aβ deposition, the B6-APPSS1 mouse model of AD, which is the only cDNA-based transgenic mouse model of AD that is in a pure, inbred C57BL/6J genetic background was used.

**Steady State Aβ Levels**

To examine the effect of A/J CSSs on Aβ metabolism, homozygous B6.A4, B6.A9 and B6.A11 mice were mated to homozygous B6-R1.40 mice. The resulting animals that were heterozygous for the A/J chromosome and
homozygous for the R1.40 transgene were subsequently backcrossed to the homozygous CSS stock strains and animals identified that were hemizygous for the R1.40 transgene and homozygous for the respective non-recombinant A/J chromosome by genotyping. The steady state levels of brain Aβ in the resulting B6.A4-R1.40, B6.A9-R1.40 and B6.A11-R1.40 as well as B6-R1.40 animals was assessed by ELISA (Figure 4-2). Notably, both B6.A9-R1.40 and B6.A11-R1.40 animals exhibited a statistically significant increase in brain Aβ levels when compared to the B6-R1.40 parent. While the B6.A4-R1.40 animals also exhibited a slight increase in brain Aβ levels, this failed to reach statistical significance. Taken together, these results suggest that there are genetic loci on A/J chromosomes 9 and 11 that elevates the levels of brain Aβ.

Aβ Deposition

Next, to examine the effect of A/J CSSs on Aβ deposition, homozygous B6.A4, B6.A9 and B6.A11 mice were mated to homozygous B6-APPPS1 mice. The resulting animals that were heterozygous for the A/J chromosome and hemizygous for the APPPS1 transgene were subsequently backcrossed to the homozygous CSS stock strains and animals identified that were hemizygous for the APPPS1 transgene and homozygous for the respective non-recombinant A/J chromosome by genotyping. B6.A4-APPPS1, B6.A9-APPPS1, B6.A11-APPPS1 and B6-APPPS1 animals were subsequently aged to 4 months of age, as this is an age at which there is abundant Aβ deposition throughout the cortex as well as more modest Aβ deposition in the hippocampus. Notably, while B6.A4-APPPS1
and B6.A9-APPPS1 mice did not exhibit differences in Aβ deposition as detected by Thioflavin S staining when compared to B6-APPPS1 mice, B6.A11-APPPS1 mice exhibited statistically significant reductions in Aβ deposition at 4 months of age (Figure 4-3).

**APP Processing**

To confirm that the effect of the A/J chromosomes was not due to an alteration in transgene expression, the levels of holo-APP and APP CTFs were analyzed in the B6.A11-APPPS1 and B6-APPPS1 animals, revealing no significant differences in either holo-APP or APP CTFs (Figure 4-4). Taken together, these results suggest that genetic loci on A/J chromosome 11 elevates steady state levels of Aβ in young animals and yet reduces Aβ deposition in older animals, similar to the A-R1.40.

**Identification of DBA/2J Genetic Loci that Alters Aβ Deposition**

A previous QTL analysis in the lab demonstrated that chromosomal loci on DBA/2J chromosomes 1, 2 and 7 were associated with reduced steady state levels of brain Aβ (Davis et al., 2005). A separate study describes the generation of a chromosome 2 congenic strain and analysis of effects on Aβ metabolism and deposition. The current studies focus on the effects of genetic loci on DBA/2J chromosomes 1 and 7 on Aβ metabolism and deposition. To facilitate this analysis, a panel of B6.D2 congenic strains that were previously generated and described were utilized (Davis et al., 2005). Two congenic strains, B6.D2.1D and
B6.D2.7, that overlapped the QTLs on mouse chromosomes 1 and 7 were identified and imported into the animal facility.

As described above, the current studies utilized two different mouse models of AD to examine the effects of the DBA/2J chromosome 1 and 7 congenic intervals on Aβ metabolism on the one hand and Aβ deposition on the other. The mating of the B6.D2 congenics to the B6-R1.40 mice provides important data regarding genetic loci that regulate the steady state levels Aβ. On the other hand, the mating of the B6.D2 congenics to the B6-APPPS1 mice focused on the identification of DBA/2J genetic loci that modify Aβ deposition.

**Steady State Aβ Levels**

To examine the effect of B6.D2 congenic strains on Aβ metabolism, homozygous B6.D2.1D and B6.D2.7D mice were mated to homozygous B6-R1.40 mice. The resulting animals that were heterozygous for the DBA/2J congenic interval and hemizygous for the R1.40 transgene were subsequently backcrossed to the homozygous congenic stock strains and animals identified that were hemizygous for the R1.40 transgene and homozygous for the respective DBA/2J congenic interval by genotyping. The steady state levels of brain Aβ in the resulting B6.D2.1D-R1.40 and B6.D2.7D-R1.40 animals as well as B6-R1.40 animals was assessed by ELISA (Figure 4-5). Notably, neither the B6.D2.1D-R1.40 or B6.D2.7D-R1.40 animals exhibited a statistically significant difference in brain Aβ levels when compared to the B6-R1.40 parent. However, as the QTL analysis suggested that the chromosome 1 and 7 locus contributed
only 4% of the variance in the levels of Aβ between the D2-R1.40 and B6-R1.40 strain. Power analysis determined a 0.1 difference in mean would require 200-300 animals per group to establish significance.

*Aβ Deposition*

Next, to examine the effect of B6.D2 congenic strains on Aβ deposition, homozygous B6.D2.1D and B6.D2.7D mice were mated to homozygous B6-APPPS1 mice. The resulting animals that were heterozygous for the DBA/2J congenic interval and hemizygous for the APPPS1 transgene were subsequently backcrossed to the homozygous B6.D2 congenic stock strain and animals identified that were hemizygous for the APPPS1 transgene and homozygous for the respective congenic interval by genotyping. B6.D2.1D-APPPS1, B6.D2.7D-APPPPS1 and B6-APPPS1 animals were subsequently aged to 4 months of age and examined for alterations in Aβ deposition (Figure 4-6). Notably, while B6.D2.7D-APPPPS1 mice did not exhibit differences in Aβ deposition as detected by Thioflavin S staining when compared to B6-APPPS1 mice, B6.D2.1D mice exhibited statistically significant reductions in Aβ deposition at 4 months of age. Taken together, these results suggest that genetic loci within the B6.D2.1D congenic interval reduce Aβ deposition.
**APP Processing**

To determine if alterations in APP processing could be responsible for the reductions in Aβ deposition seen in B6.D2.1D-R1.40 animals, the levels of holo-APP as well as the proteolytic processing products of APP, CTF_β (representing amyloidogenic processing of APP) and CTF_α (representing nonamyloidogenic processing of APP), were analyzed by western blot (Figure 4-7). APP processing in the B6.D2.1D-APPPS1 animals was compared to that of the B6-APPPS1. Notably, the levels of holo-APP, as well as CTF_β and CTF_α levels were unaltered between the two strains.

**Analysis of B6.D2.1D Congenic Interval**

To identify possible candidate genes that may be responsible for differences in Aβ deposition observed in the B6.D2.1D animals the portion of mouse chromosome 1 was examined in more detail. This region of chromosome 1 is syntenic to the human chromosome 1q44. The D1 congenic interval falls between rs3688785 (154378698 bp) and rs29609526 (19134686 bp) and is 43 Mb in size. The congenic region contains 831 genes and 94 of these genes contain coding-nonsynonymous polymorphisms. Of the 831 genes, 16 genes have been previously associated with AD, the most recognizable of these are *Ncstn*, *Psen2*, and *Apoa2*. *Psen2* contains noncoding polymorphisms, *Ncstn* contains noncoding polymorphisms as well as coding-synonymous polymorphisms, and *Apoa2* contains both coding and noncoding polymorphisms. Of the 16 genes associated with AD, 3 genes contain coding-nonsynonymous
polymorphisms, namely Camk1g, Crp and Soat1. Further studies will be required to assess the relevance of these different candidate genes in regulating Aβ deposition.

**DISCUSSION**

Studies analyzing both fraternal and identical twins has revealed that genetic factors likely account for a majority of the risk for AD. However the identification of these genes has proven exceedingly difficult. Genome wide association studies have shown there are likely numerous genes that influence AD risk, most of which have very small effects on overall risk, but together may have substantial effects with a given population (Bertram et al., 2008; Coon et al., 2007). Further complicating these human genetic studies, is the fact that current evidence suggests that there are a large number of environmental factors that also modify AD risk and are difficult to control for. The current strategy is to look at extremely large sample sizes in case-controls studies to identify all of the genes of small effect size.

Mouse models of AD provide a unique experimental platform to effectively identify genetic modifiers of AD phenotypes. First, all individual animals in an inbred strain are genetically identical therefore large numbers of animals can be generated to detect very small phenotypic differences. Second, the environment of a mouse is highly regulated thus reducing potential confounds due to environmental influences. Third, there are a number of models of AD that recapitulate key aspects of AD phenotypes. The current study used a unique
mouse model of AD, the R1.40 mouse model, that has been backcrossed into 4
different inbred strains resulting in identical insertion of the transgene in four
defined genetic backgrounds. Fourth, there are unique genetic resources
available in the mouse, including chromosome substitution strains and congenic
strains that can be utilized to specifically identify genetic loci that modify AD
phenotypes.

To examine the effect of genetic background on APP processing, Aβ
metabolism and Aβ deposition four different congenic R1.40 strains (B6-R1.40,
D2-R1.40, 129S1-R1.40 and A-R1.40) were generated. These studies
demonstrate that B6-R1.40 animals have high levels of steady state Aβ in young
animals, with Aβ deposition occurring at 13 months; while the 129S1-R1.40 and
the D2-R1.40 lines have low levels of steady state brain Aβ early in life with Aβ
deposition delayed until 24 months (Lehman et al., 2003a). Most notably, the A-
R1.40 strain exhibited steady state brain Aβ levels that was significantly higher
than all of the other strains but the A-R1.40 failed to exhibit any Aβ deposits,
even at advanced ages. These results suggest that genetic loci within the A/J
strain block Aβ deposition within the normal lifespan of a mouse.

The finding that A-R1.40 animals fail to exhibit any Aβ deposition despite
elevated levels of brain Aβ in young animals is consistent with previous findings
in the TgCRND8 mouse model of AD (Sebastiani et al., 2006). Sebastiani et al.
used F2 QTL mapping to identify three chromosomes (4, 9, and 11) associated
with reduced Aβ deposition in the A/J strain. To specifically examine whether
genetic loci on these chromosomes could be playing a role in altering Aβ
metabolism and deposition, chromosome substitution strains, in which these A/J chromosomes were present in a C57BL/6J background, were mated to two different mouse models of AD. These studies confirmed that genetic loci on A/J chromosome 11 reduce Aβ deposition. One of the unique advantages of the CSSs, is that sub-interval congenics can subsequently be generated to assess the role of genetic loci on specific parts of the chromosome identified and these experiments are underway for A/J chromosome 11. Mouse chromosome 11 has a mapped length of 104 cM (122 Mb) and has an estimated 2082 refseq genes (http://www.informatics.jax.org/mgihome/other/mouse_facts1.shtml, http://genome.ucsc.edu/ respectively). Mouse chromosome 11 has homology to regions of human chromosomes 1, 2, 5, 7, 16, 17 and 22 (http://www.ncbi.nlm.nih.gov/projects/homology/maps/). There are two important candidate genes located within chromosome 11 angiotensin converting enzyme (ACE) and microtubule-associated protein tau (MAPT). ACE has been associated with AD in 18 human association studies (alzgene.org). There is some experimental evidence that ACE can degrade Aβ (Hu et al., 2001; Zou et al., 2007). MAPT has been associated with AD in 15 human association studies (alzgene.org). The hyperphosphorylated form of MAPT (tau) aggregates to form neurofibrillary tangles, one of the most prominent pathological hallmarks of AD. Both of these genes contain intronic single nucleotide polymorphisms between the A/J and C57BL/6J strains (http://www.informatics.jax.org/strains_SNPs.shtml).
Another important finding of these studies is the identification of chromosomal regions from the DBA/2J mouse strain that modifies Aβ deposition. Previous studies revealed reduced steady state Aβ levels and delayed Aβ deposition in the D2-R1.40 strain. A previous QTL mapping study between the C57BL/6J and DBA/2J strains suggested an association with reduced Aβ and the distal region of mouse chromosome 1, the proximal portion of chromosome 2 and the medial portion of mouse chromosome 7. Studies on chromosome 2 identified C5 as a gene involved in reduced Aβ deposition. Notably, through the use of a congenic strain, in which the distal portion of DBA/2J mouse chromosome 1 was introduced into a C57BL/6J genetic background, the present study demonstrated that there were genetic loci within this portion of chromosome 1 that reduced Aβ deposition. However, similar studies with a congenic strain for the medial portion of DBA/2J chromosome 7 failed to identify genetic loci in this interval that altered Aβ metabolism or Aβ deposition. Closer examination of the congenic region on mouse chromosome 1 reveals the presence of several genes that have been previously associated with AD. For example, the C-reactive protein (CRP) gene encodes an inflammatory molecule that has been associated with pathology in human AD brains (Duong et al., 1997; Iwamoto et al., 1994). Furthermore, a human genetic association study examining the CRP gene suggested a positive association between CRP polymorphism and AD. However, the exact significance of this finding remains unclear, as the association between CRP and AD was not replicated in two other independent studies (Flex et al., 2004; Giedraitis et al., 2009; van Oijen et al., 2007). Notably,
two genes that encode components of the $\gamma$-secretase complex are located within this interval, namely nicastrin (NCSTN) and presinilin 2 (PSEN2). Both of these genes have been associated with AD in genetic association studies (Brookes et al. 1997; Confaloni et al., 2003; Dermaut et al., 2002; Helisalmi et al., 2004; Liu and Jia, 2008; Ma et al., 2009; Piscopo et al., 2006; Zhong et al., 2009). This association is potentially significant, as mutations in two different components of the $\gamma$-secretase complex, namely both PSEN1 and PSEN2 have been shown to cause AD. Next, the expression of the regulator of G-protein signaling 4 (RGS4) gene is within the congenic interval and is down-regulated in AD (Emilsson et al., 2006; Muma et al., 2003). Several other genes, including the Fc fragment of IgE, high affinity I, receptor for gamma subunit (FCER1G), 11-beta-hydroxysteroid dehydrogenase, type 1 (HSD11B1), poly (ADP-ribose) polymerase 1 (PARP1), POU domain, class 2, transcription factor 1 (POU2F1), and sterol O-acyltransferase 1 (SOAT1) have all been associated with AD in human genetic studies (de Quervain et al., 2004; Infante et al., 2007; Lamsa et al., 2007; Taguchi et al., 2005; Wollmer et al., 2003). Future studies are required to examine the role of these genes in the observed differences in A$\beta$ deposition.

AD is a complex disease, involving a large number of both genetic and environmental modifiers of disease that likely together influence disease risk. Using defined transgenic mouse models of AD and unique genetic mapping resources available in the mouse, these studies identified two different chromosomal loci that influence A$\beta$ deposition. Future studies will further narrow
the intervals identified as well as focus on candidate gene analysis to ultimately identify the gene(s) responsible.
A

B6-R1.40  D2-R1.40  129-R1.40  AJ-R1.40

holoAPP

97.4kDa

βCTF

14.3kDa

αCTF

6.2kDa

B

pmol Aβ40-42/g brain

n=4

B6 v. AJ  P < 0.05
B6 v. D2  P < 0.05
AJ v. 129  P < 0.01
AJ v. D2  P < 0.001

One-way ANOVA w/ Tukey-Kramer Correction

C

D6-R1.40  A-R1.40

129S1-R1.40  D2-R1.40

Amyloid Burden

B6-R1.40  A-R1.40  129S1-R1.40  D2-R1.40
Figure 4-1: Analysis of the congenic R1.40 mouse model of AD

A. APP processing at 28 days in the congenic R.140 strains. CHAPs brain extracts were analyzed for by Western blot using antibody 369 specific for holo-APP and APP CTFs. The levels of holo-APP as well as CTF β were not different between the four different strains. B. Steady state Aβ levels at 28 days in congenic R1.40 strains. Guanidine brain extracts were analyzed for steady state levels of Aβ1-40 by ELISA. The B6-R1.40 strain exhibited significantly higher steady state levels of brain Aβ compared to the D2-R1.40 strain (p<0.05) and the level of Aβ in the 129S1-R1.40 strain was intermediate (Lehman et al., 2003a). The A-R1.40 strain exhibited the highest levels of steady state brain Aβ when compared to either the B6-R1.40 strain (p<0.05) or the other two strains. C. Aβ deposition in the congenic R1.40 lines at 24 months. The cortex of paraffin sections (2 sections/animal) were analyzed for Aβ deposition using the 6e10 antibody, which is specific for Aβ1-17 and Image J to identify plaque area. While not statistically significantly different, the B6-R1.40 strain exhibits extensive Aβ pathology, while the 129S1-R1.40 and D2-R1.40 lines strains exhibit a low, but detectable amount of Aβ deposition, and the A-R1.40 strain fails to exhibit Aβ pathology at any age examined (out to 28 months) (p=0.29 by one way ANOVA with Tukey’s multiple comparison test).
Figure 4-2: Steady state $A\beta$ levels are increased in the B6.A-R1.40 consomic strains compared to the B6-R1.40 controls. B6-R1.40, A-R1.40 and B6.A11-R1.40 animals were sacrificed at 28 days. Steady state levels of $A\beta$ were analyzed by ELISA. Presence of the R1.40 transgene on the A/J background results in significantly higher $A\beta$ levels compared to the B6-R1.40 strain ($p<0.05$). Likewise, B6.A9-R1.40 ($p<0.01$) and B6.A11-R1.40 ($p<0.001$) animals have significantly higher levels of steady state $A\beta$ levels at 28 days compared to the B6-R1.40 controls.
Figure 4-3: Brain Aβ deposition reduced in the presence of A11 chromosome. Animals were sacrificed at 4 months of age and examined for Aβ deposition using 6e10 antibody and Thioflavin S staining. The left panel represents 6e10 staining in B6-APPPS1, B6.A4-APPPS, B6.A9-APPPS1, and B6.A11-APPPS1 animals. The right panels represent Thioflavin S staining in B6-APPPS1, B6.A4-APPPS1, B6.A9-APPPS1, and B6.A11-APPPS1. Cryosections were analyzed (2 sections/animal) for the percent area of Aβ deposit in the somatosensory cortex using Image J. The B6.A11-APPPS1 animals were the only strain to show a reduction in Aβ deposition compared to the B6-APPPS1 control animals (p<0.001 by one way ANOVA with Tukey’s multiple comparison test).
Figure 4-4: APP processing is unaltered in the B6.A11-APPPS1 consomic animals. B6.A11-APPPS1 and B6-APPPS1 animals were sacrificed at 2 months for APP processing. CHAPs brain extracts were analyzed for Holo-APP and APP CTFs by Western blot using antibody 369. There is no significant difference in the level of holo-APP nor the levels of CTF_α or CTF_β between strains as determined by a one way ANOVA with Tukey’s multiple comparison test.
Figure 4-5: B6.D2.1D and B6.D2.7D congenic regions are not associated with reduced steady state Aβ levels. B6-R1.40, D2-R1.40, B6.D2.1D-R1.40 and B6.D2.7D-R1.40 animals were sacrificed at 28 days. Steady state Aβ levels were analyzed by ELISA. Presence of the R1.40 transgene on the DBA/2J genetic background results in a significant reduction of steady state Aβ levels at 28 days compared to the B6-R1.40 strain (p<0.05). However, the B6.D2.1D-R1.40 and B6.D2.7D-R1.40 animals have steady state Aβ levels comparable to the B6-R1.40 controls.
Figure 4-6: Aβ deposition reduced in B6.D2.1D congenic animals. B6.D2.1D-APPPS1 and B6.D2.7D-APPPS1 animals were sacrificed at 4 months of age and examined for Aβ deposition using 6e10 antibody (left panel) and Thioflavin S staining (right panel). Cryosections were analyzed (2 sections/animal) for the percent area of Aβ deposits in the somatosensory cortex using Image J. The B6.D2.1D-APPPS1 strain exhibited a significant reduction in Aβ deposition compared to the B6-APPFS1 controls (p<0.0001 by a one way ANOVA with Tukey’s multiple comparison test).
Figure 4-7: APP processing is unaltered in the B6.D2.1D congenic strain.

B6-APPSS1 and B6.D2.1D-APPSS1 animals were sacrificed at two months of age. CHAPs extracts were analyzed by Western blot using the antibody 369 specific for holo-APP and APP CTFs. There is no significant difference in the level of holo-APP, CTF\_\textalpha, or CTF\_\textbeta, between strains as determined by a one way ANOVA with Tukey’s multiple comparison test.
Chapter 5: Summary and Future Directions
Summary

Despite being first described just over a century ago, it is only within the last 25 years that biochemical and genetic studies have provided insight into the pathogenesis of Alzheimer’s disease. Advancements in the field include the discover of Aβ and its precursor APP as well as the role of the α-, β-, and γ-secretases in the proteolytic processing of APP into Aβ; the identification of mutations in families with early onset familial AD that were shown to alter Aβ profiles; and the identification of APOE, a protein involved in Aβ metabolism, as a risk factor for late onset familial AD. Based on the exclusivity of Aβ deposition with AD and genetic evidence supporting the role of Aβ as disease causative, much of the field has focused on the production and clearance of Aβ in the brain as the key to understanding the disease.

However, many of the genetic risk factors associated with Alzheimer’s disease remain unknown. Identification of these genes has been difficult for several reasons. First, is the need for very large sample sizes due to environmental modifiers, heterogeneity, epistasis and population stratification. Second, are special considerations due to the type of study being conducted. For example, candidate gene studies rely on a previous association with the disease, while GWAS have difficulty reproducibly identifying genes unless they have a large effect size. Many of these difficulties can be overcome through the use of the mouse model in determining modifiers of AD pathogenesis.

The main objectives of this thesis work was to investigate the effects of genetic background on APP processing, Aβ metabolism and Aβ deposition as
well as identify genetic loci responsible for the observed heritable differences in
$A\beta$ metabolism and $A\beta$ deposition between congenic R1.40 lines. Through better
understanding of factors, both genetic and environmental, that can alter $A\beta$
levels; the hope is to uncover pathways that can be manipulated to alter the
disease course.

**Genes and Environment Interact to Alter $A\beta$ Levels.**

Studies have demonstrated AD is a complex, heterogeneous disorder with
numerous postulated genetic and environmental risk factors (Bertram et al.,
2008). Several lines of evidence support a hypercholesterolemic diet as a risk
factor for AD. First, genetic evidence supports that the $\varepsilon4$ allele of APOE, the
major cholesterol carrier in the brain, increases risk for AD (Corder et al., 1993).
Second, atherosclerosis, a disease of arterial build-up of fatty materials such as
cholesterol, is a risk factor for AD (Hofman et al., 1997; Honig et al., 2005).
Third, *in vitro* studies have found that cholesterol levels can regulate APP
processing and $A\beta$ generation (Fassbender et al., 2001; Frears et al., 1999;
Simons et al., 1998).

However, epidemiological studies have had mixed findings regarding an
association between altered cholesterol metabolism and AD risk as well as the
efficacy of cholesterol lowering drugs in reducing AD risk (Anstey et al., 2008;
McGuinness et al., 2010). Closer examination of these studies reveals sample
sizes too small to identify risk factors of small effect size and sampling from very
different homogenous populations that likely vary in allele frequency. These factors likely contribute to the inconsistency between studies.

While traditionally animal studies have been able to overcome these problems, mouse models of AD have also had conflicting findings regarding the effect of hypercholesterolemic diet on AD risk. A review of the literature has found that these studies vary in several ways. First, there is variation in the transgene used to create the mouse model of AD. They vary in the familial human AD mutation contained within the construct as well as the promoter used to drive expression. Second, studies vary in the composition of the diet as well as the onset and duration of exposure. Finally, the genetic background of the mouse models of AD varies.

The congenic R1.40 mouse model of AD is a superior model to test the effects of a high-fat/high-cholesterol (HF/HC) diet because the R1.40 transgene is identically inserted into three different genetic backgrounds. Animals are fed the same diet and housed in the same facility therefore effects of HF/HC diet on APP processing and Aβ metabolism are due to allelic differences between background strains. Analysis of the effect of a HF/HC diet on the congenic R1.40 mouse model of AD revealed that alteration in steady state Aβ levels was based on genetic background. While the DBA/2J and 129S1/SvImJ strains showed alterations in body weight and serum cholesterol due to the HF/HC diet, they did not have altered levels of brain Aβ (Figure 2-1). In contrast, the C57BL/6J animals had increases in body weight, serum cholesterol and brain Aβ on the HF/HC diet that appear to be due to strain specified alterations in gene
expression (Figure 2-5). In support of our findings, other studies found that the transgenic mouse models of AD on a C57BL/6J background that were fed HF/HC diet, regardless of transgene or diet, showed elevated brain Aβ (Fitz et al., 2010; Levin-Allerhand et al., 2002; Li et al., 2006; Hooijmans et al., 2007).

These results provide the first experimental evidence that the affects of a postulated environmental risk factor for AD, dietary fat and cholesterol, are dependent upon genetic background. This study provides further support for a complex interplay between genetic and environmental risk factors as responsible for AD. This study suggests that specific genes in the C57BL/6J strain allow for a HF/HC diet to alter brain Aβ.

Identification of a Gene Associated With Altered Aβ Levels.

The heritability of AD is 79%, however the majority of genes associated with AD have yet to be identified. Unlike EOFAD where the genetic factors are disease causative, LOFAD genes alter disease risk (Tanzi et al., 1999). This study reveals that the mouse model of AD is an effective way to identify risk factors for LOFAD, in this case genes that alter AD pathology.

QTL mapping studies between the B6-R1.40 and D2-R1.40 lines revealed a significant association with reduced steady state Aβ levels and a region of mouse chromosome 2 (Ryman et al., 2008). Analysis of B6.D2-R1.40 animals, B6-R1.40 animals containing a DBA2/J segment of chromosome 2 corresponding with the region identified in mapping studies, found reduced Aβ deposition (Figure 3-4). This suggested the presence of a genetic risk factor of
AD involved in Aβ metabolism within the chromosome 2 segment. Evaluation of the ~25 Mb region revealed the presence of a loss of function mutation in the C5 gene. C5 is a component of the complement cascade, part of the body’s innate immunity. The complement system and C5 have been implicated in AD (Afagh et al., 1996; Aikayama et al., 2000; Bradt et al., 1998; Fonseca et al., 2004; Fonseca et al., 2009; Loeffler et al., 2008; Maier et al., 2008; Matsuoka et al., 2001; Rogers et al., 1992; Wyss-Coray et al., 2002; Yang et al., 2000; Zanjani et al., 2005). Studies have demonstrated that inhibition of the C5a receptor (C5aR) results in increased Aβ deposition (Fonseca et al., 2009). To test if alterations in Aβ in the B6.D2-R1.40 animals is mediated through C5a-C5aR signaling, two AD models (R1.40 and PS1APP) deficient for the C5aR were analyzed for Aβ deposition. These studies support previous finding that altering the C5 signaling pathway reduces Aβ deposition (Figure 3-7, Figure 3-8). This study is unique in that it demonstrates both the absence of the protein (C5a) as well as the receptor (C5aR) alters Aβ deposition and confirms the findings in two different mouse models of AD.

This study provides a pathway for further examination in the human. The finding of the current study in combination with a finding that the C5aR antagonist PMX205 inhibits C5-C5aR signaling with out altering C1q and C3 function have implications for AD therapy (Ager et al., 2010).
Genetic Background Alters Aβ Levels.

Previous studies in the congenic R1.40 mouse model of AD examined the effect of genetic background on APP processing, Aβ metabolism and Aβ deposition and revealed alterations between strains. The R1.40 transgene in the C57BL/6J background results in elevated Aβ levels and age-related deposition. In contrast, the 129S1/SvImJ and DBA/2J background had reduced Aβ levels and deposition (Lehman et al., 2003a). This study expands the previous findings by examining APP processing, Aβ metabolism and Aβ deposition in the A-R1.40 line (Figure 4-1). Surprisingly, the A/J background had the highest Aβ levels but an absence of Aβ deposition. It was previously believed an increase in Aβ levels corresponded with increase Aβ deposition and vise versa. This is the first time a study has shown steady state Aβ levels are not correlated to Aβ deposition.

This thesis determined the insertion sites of the R1.40 transgene into chromosome 13 are similar in all R1.40 lines, suggesting differences in phenotype between strains are due to allelic variation between genetic backgrounds and not due to passenger loci. The results demonstrate that Aβ metabolism is under genetic control in the mouse. They suggest the presence of loci in the DBA/2J and 129S1/SvImJ strains that maintain low steady state Aβ levels. They also suggest the presence of loci on the A/J strain that prevent Aβ deposition despite elevated steady state Aβ levels. This study also illustrates the complexity in designing mouse studies, providing evidence that genetic background should be a consideration in planning experiments as well as in the analysis of results.
There are several genetic tools available in the mouse to identify these genetic modifiers of Aβ metabolism including QTL analysis, chromosome substitution strains (CSS) and congenic lines. Previous QTL mapping studies between the C57BL/6J and A/J strains in the TgCRND8 mouse model of AD revealed associations with brain Aβ and mouse chromosome 4, 9 and 11 (Sebastiani et al., 2006). B6.A chromosome substitution strains (CSS), a strain in which a single chromosome from the C57BL/6J strain is replaced by the corresponding A/J chromosome, with the R1.40 or APPPS1 transgene (B6.A4, B6.A9 and B6.A11) were examined. Only chromosome 11 was associated with reduced Aβ deposition (Figure 4-3). This finding suggests the presence of a gene(s) within chromosome 11 that modify Aβ deposition.

QTL mapping between the B6-R1.40 and D2-R1.40 lines revealed associations with reduced steady state Aβ levels and mouse chromosome 1 and 7 (Ryman et al., 2008). To confirm these associations B6.D2-R1.40 and B6.D2-APPPS1 congenic animals were analyzed for Aβ levels. These animals contain an APP causing transgene (R1.40 or APPS1) as well as a single chromosomal segment from the C57BL/6J strain corresponding to the peak in the association study replaced by the corresponding DBA2/J region. The chromosome 1 congenic region was associated with reduced Aβ deposition (Figure 4-6). These studies provide evidence that this region contains a gene(s) involved in altering Aβ deposition.

Using the congenic R1.40 mouse model of AD the chromosomal regions associated with reduced Aβ deposition were narrowed to mouse chromosome 11
in the A/J strain, a region of mouse chromosome 1 in the DBA2/J strain and a
gene on mouse chromosome 2 in the DBA2/J strain. These studies illustrate the
effectiveness of the mouse model in identifying risk factors for AD pathogenesis.

**Future Directions**

**Characterization of Genetic Loci That Alter Effects of Cholesterol Metabolism**

The study in chapter 2 revealed that difficulties in reproducibly identifying
environmental risk factors of disease might be due to genetic background. Allelic
variation within the C57BL/6J genetic background confers risk for altered Aβ
metabolism in the presence of HF/HC diet. This study leads to compelling
questions regarding the mechanism by which Aβ is altered. Future studies
proposed here could address these questions and further define how cholesterol
interacts with genetic factors to modify AD risk.

First closer examination of the genes alternatively expressed in the
C57BL/6J strain in the presence of a HF/HC diet is required. The most likely
candidate to examine is the *THOP1* (*metallopeptidase EC 3.4.24.15*) gene. Both
the human AD brain and the transgenic AD mouse brain have increased
expression of the *THOP1* gene and exhibit THOP1 protein association with Aβ
deposits and neurofibrillary tangles (Conn et al., 1996; Pollio et al., 2008).
Studies in cell culture revealed transfection of cells with THOP1 reduced the
levels of Aβ in the media and that co-transfection with both THOP1 and Aβ
resulted in degradation of Aβ that was ameliorated by co-transfection of anti-
sense THOP1 and Aβ (Koike et al., 1999; Pollio et al., 2008; Yamin et al., 1999). While in vitro evidence supports a role of THOP1 in Aβ degradation, it is important to examine the effect of the THOP1 on steady state brain Aβ in vivo as well as in the presence of HF/HC diet. One way to do this is to alter gene expression in a mouse model of AD exposed to a HF/HC diet. Studies have shown success in using the adenovirus to either over express a gene or knock down gene expression in mouse models (Chiba et al., 1999; Feng et al., 2004; Nijstad et al., 2011; Ryan et al., 2010; Wang et al., 2010). Mice would be examined for levels of Aβ and Aβ deposition.

To examine the relevance of these findings in humans, it is also important to examine the 159 genes identified in this study in human studies looking at cholesterol and AD. A number of investigators have assembled collections of DNA, serum cholesterol and tissue samples from a large number of AD patients throughout the world. Studies examining the effect of serum cholesterol on AD have had contradictory findings. Through collaborations between these investigators, the genes identified in this study should be examined for alterations in gene expression between individuals with high serum cholesterol with AD versus high serum cholesterol without AD. This would determine if allelic variation within the human population modifies the effect of cholesterol on AD risk.

The second set of experiments would involve further examination of gene-environment interactions. This study showed that Aβ levels at 28 days are increased in the C57BL/6J strain in the presence of HF/HC diet. However what does this mean for Aβ deposition? Do C57BL/6J animals have deposition earlier
in life when exposed to a HF/HC diet? Is the plaque load more robust? Studies in humans have shown high cholesterol mid-life is associated with increased \( A\beta \) deposition. Therefore, 6 month old B6-R1.40 animals would be exposed to a HF/HC or control diet for 6 months. At sacrifice, brains would be paraffin embedded, sectioned, stained with an antibody against \( A\beta \) (6E10) and \( A\beta \) deposition quantified.

Another important study to conduct is to repeat the eight week exposure of HF/HC diet in four week old A-R1.40 animals to determine the effects of HF/HC diet on \( A\beta \) levels. This body of work has shown that the A/J strain has increased levels of \( A\beta \) compared to the C57BL/6J strain. Studies have shown that the A/J strain is an obesity resistant strain while the C57BL/6J confers susceptibility to obesity and hypercholesterolemia (Surwit et al., 1988). Is the A/J genetic background hypersensitive to a HF/HC diet therefore increasing brain \( A\beta \) levels? Will the A/J animals get \( A\beta \) deposition? Are similar genes up- and down-regulated in the A/J strain in the presence of the HF/HC diet?

**Examination of Mechanism by which C5 Clears \( A\beta \)**

The study in chapter 3 identified a locus associated with reduced \( A\beta \) deposition, namely \( C5 \). Future studies will include closer examination of \( C5 \) and its effect on \( A\beta \) metabolism. Reduction of \( A\beta \) deposition through inhibition of C5a-C5aR signaling is thought to be mediated through microglia (Fonseca et al., 2009). Preliminary examination of the B6.D2.2D-R1.40 and C5aRKO-R1.40 animals did not reveal changes in microglial number or morphology. However,
microglia may still be involved. To determine if there is an alteration in the phagocytic activity of microglia in the absence of the C5-C5aR pathway that maybe responsible for alterations in Aβ deposition, microglia from mouse models of AD both deficient and suffient for C5 and C5aR would be examined. Previous studies have shown that proinflammatory cytokines (IL-1β, TNFα, INFγ, MCP-1 and CD40L) inhibit microglial phagocytosis and inhibit microglial degradation of Aβ (Koenigsknecht-Talboo and Landreth 2005; Yamamoto et al., 2008). Anti-inflammatory cytokines (IL-4, IL-10 and COX) have been shown to ameliorate these effects and promote Aβ degradation. Brains of R1.40 animals with or without the C5-C5aR signaling pathway would be examined for expression of inflammatory markers (IL1β, TNFα, INFγ, IL4, IL10 and MCP-1) by qRT-PCR to determine if the cytokine milieu is conducive for Aβ uptake and degradation in the absence of the signaling pathway. To test if there is varied phagocytic capacity of the microglia, primary microglia from B6.D2.2D-R1.40, C5aRKO-R1.40 and B6-R1.40 animals would be exposed to fluorescent microspheres and quantified for internalization of spheres. Likewise, the ability of the R1.40 microglia to phagocytose fibrillar Aβ in the presence or absence of C5-C5aR signaling would be examined in culture using a phagocytic assay described by Koenigsknecht and Landreth (2004).

Characterization of A/J
The study in chapter 4 confirmed that genetic background modifies Aβ metabolism and for the first time showed that Aβ levels are not correlated to Aβ deposition. This study leads to compelling questions regarding the A/J strain. First, the A/J strain has no Aβ deposition, but studies have shown Aβ can go down another path resulting in Aβ oligomers (Aβo). Therefore, it is important to determine if there are alterations in Aβo levels between the B6-R1.40 and A-R1.40 strains. To test this would require collaboration with a lab that can successfully assay Aβo. Brain homogenates from congenic R1.40 animals of all strains would be analyzed at 6 months. Second, how does the pathological profile in the A/J strain correlate to cognition? Studies have shown the B6-R1.40 animals, which have high levels of Aβ early in life and deposition late in life, have behavioral deficits (Hock 2009). While behavior cannot be compared between strains due to physical constraints unique to each strain it is important to examine changes in cognition within the A/J strain in the presence or absence of the R1.40 transgene. Finally, what are the genes responsible for decreased Aβ deposition in the A/J strain? Studies examining B6.A4-APP5S1, B6.A9-APP5S1 and B6.A11-APP5S1 consomic animals for Aβ deposition revealed an association with mouse chromosome 11. Future studies in this model will include narrowing the region containing the modifier of deposition. The lab is currently generating a congenic panel in which proximal, medial and distal regions of the A/J chromosome are introgressed into B6-APP5S1 animals. 4 month old animals from each congenic strain will be examined for Aβ deposition as described above. Once a region is identified that is associated with reduced Aβ
deposition, a candidate gene approach will be used to identify gene(s) associated with altered Aβ deposition.

**Conclusion**

Alzheimer’s disease is a complex disease involving both genetic and environmental risk factors of disease. Many of the genetic risk factors associated with the disease have yet to be identified. This thesis illustrates the value of the mouse model in identifying risk factors for AD, moving from genetic differences between strains (congenic R1.40 lines) to chromosome (CSS) to chromosome region (congenic lines) and finally identification of a gene. It confirms that genetic background affects Aβ metabolism. It provides evidence that C5 is one of the genes involved in altering brain Aβ levels. It shows for the first time that increases in steady state Aβ levels are not always correlated to increased Aβ deposition. Finally, it shows that environmental factors, such as diet, can alter gene expression that results in altered Aβ metabolism.


