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Phenotyping Double Transgenic Mouse Models of Alzheimer's that Express Human APP and ApoE3 or ApoE4

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by

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Each person whose signature appears below certifies that this thesis in his/her opinion is adequate, in scope and quality, as a thesis for the degree Doctor of Philosophy.

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ABBREVIATIONS

AD	Alzheimer's disease
apoE	Apolipoprotein E
APOE	Apolipoprotein E gene
E2	*APOE2*
E3	*APOE3*
E4	*APOE4*
PDAPP	Platelet-derived growth factor promoter
APP	Amyloid precursor protein
APP	Amyloid precursor protein gene
KO	Knockout mouse
KI	Knockin mouse
DAPI	4',6-diamidino-2-phenylindole
NFT	Neurofibrillary tangle
NT	Neuropil thread
FAD	Familial AD
PSEN	Presenilin gene
TG	Transgenic
TBI	Traumatic brain injury

ABSTRACT OF THE THESIS

Phenotyping Double Transgenic Mouse Models of Alzheimer's that Express Human APP and ApoE3 or ApoE4

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Doctor of Philosophy, Graduate Program in Clinical Psychology
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Dr. Richard Hartman, Chairperson

Alzheimer's disease (AD) is a neurodegenerative disorder that causes progressive cognitive and behavioral problems resulting from a build-up of amyloid-beta (Aβ) plaques in the brain. The degree of neuropathology is partially related to apolipoprotein E (apoE), a fat-binding protein involved in transporting cholesterol to neurons. The *APOE* gene, which codes for the production of apoE, has several alleles in humans, including *APOE3* (E3; the most common) and *APOE4* (E4; which is associated with a high risk for developing AD).

Transgenic mouse models of AD are commonly used to study the neuropathological processes behind the development of Aβ plaques, neurodegeneration, and associated behavioral deficits. The current study was designed to determine whether expression of E4 in a transgenic mouse model of AD (the PDAPP mouse) alters these processes in a way that replicates the effects observed in humans with E4. The brains of male PDAPP x *APOE3* mice (PDAPP:E3; n=5) were compared to those of PDAPP x *APOE4* mice (PDAPP:E4; n=4) by staining tissue sections with HJ3.4, thioflavin-S, and 4',6-diamidino-2-phenylindole (DAPI) to quantify diffuse Aβ, fibrillar Aβ, and cellular count, respectively.

PDAPP:E4 mice had more total Aβ in the CA1 of the hippocampus and dorsal cortex. Also, a trend suggested PDAPP:E4 mice had a lower cellular density in the CA1 and dorsal cortex than PDAPP:E3 mice. Importantly, more Alzheimer's-like neuropathology was generally associated with worse behavioral deficits. Since these results replicate aspects of human AD (plaque load, lower cellular density, and behavioral deficits), the expression of human *APOE* in transgenic mice may improve their use as a model system for understanding the processes involved in the development of AD and therapeutic strategies for dealing with the disease.

CHAPTER ONE

SPECIFIC AIMS

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by global cognitive decline involving memory, orientation, judgment and reasoning. AD is the most common cause of dementia, a decline in mental ability due to a brain disease or injury, in the elderly. In 2014, the Alzheimer's Association estimated that 5.2 million Americans had AD (*2014 Alzheimer's Disease Facts and Figures*, 2014). In familial AD (FAD, or early-onset AD), which is directly caused by an autosomal dominant gene mutation, an abnormal variation of the amyloid precursor protein gene (*APP*) gets cleaved by β- and γ-secretase, releasing a free Aβ peptide from the protein. These Aβ peptides aggregate, which lead to the accumulation of Aβ plaques in the brain, which eventually causes neuronal dysfunction in the brain. This form of AD accounts for about 1-5% of all cases of AD (Demattos, Bales, Paul, & Holtzman, 2000).

In the more common ("sporadic", or late onset) form of AD, genes can also influence the risk of developing the disease. The most-studied gene of susceptibility for sporadic AD is the *APOE* gene, which codes for the protein apolipoprotein E (apoE). *APOE* plays a role in the pathogenesis of late-onset AD, and has three major alleles: *APOE2* (E2), *APOE3* (E3), and *APOE4* (E4). Alleles are alternative forms of a gene that arise by a mutation and are found on the same place on the chromosome. Each individual carries two copies of *APOE*, one from each parent. The likelihood of someone developing AD is highly dependent upon which of these three alleles they carry (Corder et al., 1993). The current study addresses E4, which is known to increase the risk and decrease the age of onset of AD, and E3, which encodes for the most common isoform

(apoE3) and acts as a neutral allele, not influencing one's risk of developing the disease by using a double-transgenic mouse model. ApoE "knockout" (KO) mice, which do not express *APOE*, had human E3 and E4 inserted into their genomes via "knockin" (KI) technology. These models address a novel way of producing double-transgenic mice, representing a "cleaner" double-transgenic mouse model compared to what is normally seen in the literature (e.g., Hartman et al., 2002). This methodology differs from what is normally seen in the literature in that human *APOE* was not placed in a random location, but in the exact location within the mouse genome from which murine *APOE* was removed. Hypothetically, this resulted in an Aβ and *APOE* interaction more similar to what is seen in humans with AD. These mice were then crossbred with PDAPP transgenic mice, the first successful AD transgenic mouse model that contained an FAD-associated mutation (Games et al., 1995).

My hypothesis is that PDAPP mice that also express human E4 will have more Alzheimer's-like neuropathology than PDAPP mice that express human E3. The objective of this study is to determine whether targeted expression of human *APOE* alters neuropathology in a transgenic mouse model of AD by establishing a baseline neuropathological and behavioral data for a new double-transgenic mouse model of AD. Neuropathological assessment of PDAPP mice expressing human E3 and E4 has consisted of thioflavin-S staining (Kelly R. Bales et al., 1999; Fagan et al., 2002; Richard E Hartman et al., 2002). Bales et al. (1999) found that the amount of fibrillar amyloid (thioflavin-S-positive material) was significantly greater in PDAPP:E4 mice than in the PDAPP:E3 mice. Fagan et al. (2002) found that PDAPP:E4 mice had a greater Aβ burden and an earlier age of onset than the PDAPP:E3 mice. Lastly, Hartman et al. (2002)

assessed the effects of TBI (traumatic brain injury) in PDAPP:E4 and PDAPP:E3 mice and only found thioflavin-S-positive Aβ (amyloid) in the PDAPP/ε4 mice. Behavioral assessments of *APOE* transgenic mice have consisted of spatial learning and memory tests (rotating holeboard, radial arm maze, and water maze) and tests of sensorimotor capabilities and emotionality (locomotor activity and elevated plus maze) (R E Hartman et al., 2001). This objective will be met by addressing the following three aims.

Aim 1: To determine whether expression of human *APOE* will alter plaque load in the hippocampus and dorsal cortex of double-transgenic mice that model aspects of AD. Hypothesis: PDAPP mice that also express human E4 will have more amyloid-beta (Aβ) plaques in their brains than PDAPP mice that also express human E3. Rationale: Humans with 1 or 2 copies of the E4 gene generally have worse Alzheimer's neuropathology than humans without E4.

Aim 2: To determine whether expression of human *APOE* will alter cellular density in the CA1 region of the hippocampus and dorsal cortex of transgenic mice that model aspects of Alzheimer's disease. Hypothesis: PDAPP mice that also express human E3 will have a lower cellular density in the CA1 region of the hippocampus and dorsal cortices than PDAPP mice that also express human E3. Rationale: Humans with 1 or 2 copies of the *E4* gene generally have more Aβ aggregates, leading to a greater cellular loss.

Aim 3: To determine whether the degree of neuropathology is correlated with behavioral deficits observed in these mice. Hypothesis: Mice with more Alzheimer's-like neuropathology (specifically Aβ plaques and/or neuronal loss) will have worse behavioral deficits. Rationale: The formation of Aβ plaques in humans is thought to ultimately lead to the neuronal dysfunction and cognitive deficits observed in AD.

CHAPTER TWO

SIGNIFICANCE

Demographics

In 2014, the Alzheimer's Association reported that AD affects 11% of people 65 years and older and 32% of people 85 years and older (*2014 Alzheimer's Disease Facts and Figures*, 2014). In 2012, the Alzheimer's Association estimated that, of those with AD, 4% are under the age of 65, 6% are 65 to 74 years of age, 44% are 75 to 84 years of age, and 46% are 85 years or older (*2012 Alzheimer's disease facts and figures*, 2012). In addition to the high prevalence of AD (number of existing cases in a population at a given time), the estimated annual incidence (rate of developing AD in a one-year period) of AD appears to increase dramatically with age. The Journal of Alzheimer Disease and Associated Disorders estimated that by the year 2050, there would be 80 new cases of AD per 1,000 people age 65 to 74, to 282 new cases per 1,000 people age 75 to 84, to 598 new cases per 1,000 people over age 85. This increasing incidence rate is due to an aging population and social and environmental factors. The increasingly high prevalence and incidence rate of this disease is concerning, emphasizing the importance of the development of effective treatments.

Neuropathology

Plaques

Amyloid plaques are a neuropathological characteristic of AD (Calhoun et al., 1998). Amyloid plaques are formed by Aβ, a 39-to-43-amino-acid-long peptide derived by the proteolytic processing of APP. When APP is cleaved by β- and γ-secretase, Aβ

gets released into the extracellular space (Figure 1; Haass et al., 1992; Martin et al., 1995; Shoji et al., 2014; Wertkin et al., 1993; Yang, Knauer, Burdick, & Glabe, 1995), normally via synaptic activity (Kamenetz et al., 2003). Another enzyme, α-secretase, serves as a neuroprotectant by cleaving the APP molecule within the Aβ peptide region, preventing the formation of Aβ plaques.

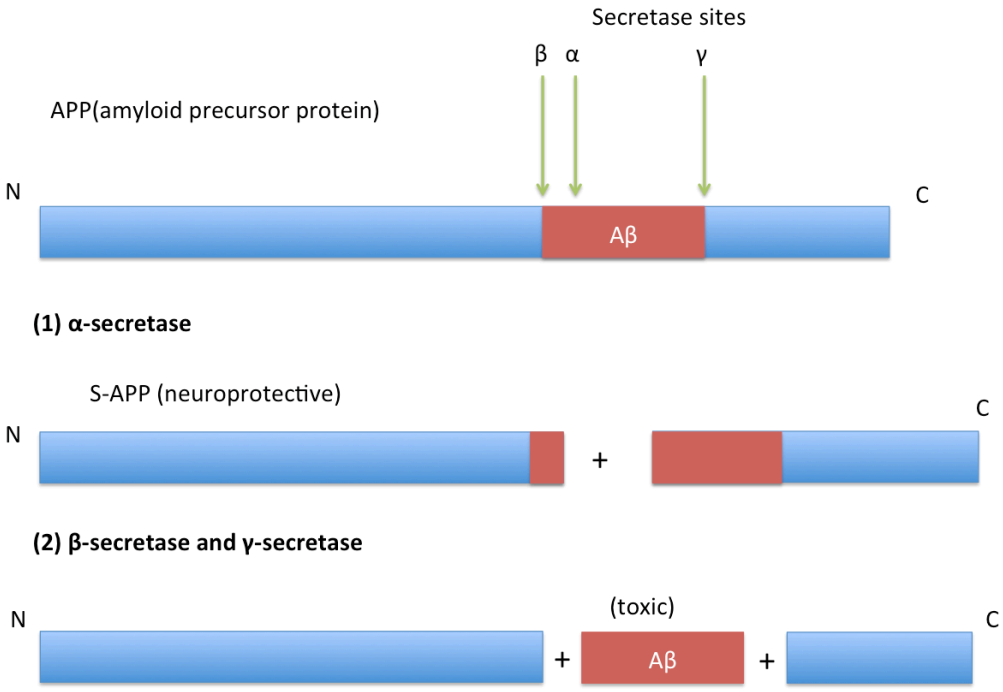

Figure 1. β- and γ-secretase cleave APP at specific sites to form toxic Aβ. Retrieved from http://commons.wikimedia.org/wiki/File:APP_cleavage_produce_toxic_Abeta.png.

The two most common isoforms of Aβ are $A\beta_{40}$ and $A\beta_{42}$, which only differ by two amino acids (Yan & Wang, 2006). $A\beta_{40}$ is a 40-residue soluble peptide, which represents the most abundant Aβ isoform, both in the normal and AD brain (Mori, Takio, Ogawarag, & Selkoe, 1992), especially in sporadic AD (Näslund et al., 1994). $A\beta_{42}$ is a 42-residue insoluble peptide, which is more closely linked to FAD (Younkin, 1998). $A\beta_{42}$

has shown to form insoluble amyloid fibrils more rapidly than the abundant $A\beta_{40}$, making it more susceptible to fibril formation in both humans and mice (Hartman et al., 2005; Jarrett, Berger, & Lansbury, 1993).

Amyloid fibrils are formed by soluble proteins that aggregate to form insoluble fibers, which are resistant to degradation (Rambaran & Serpell, 2008). Amyloid is dominated by a β-pleated sheet structure, which consists of the protein being folded in a certain way that gives it a very stable structure (Rambaran & Serpell, 2008). This process is also seen in the production of spider silk, where soluble proteins are converted to "stronger than steel" insoluble fibers (Kenney, Knight, Wise, & Vollrath, 2002). The spider silk also takes on a β-pleated sheet structure (Kenney et al., 2002). $A\beta_{42}$ is particularly known to be neurotoxic (Elder, Sosa, & Gasperi, 2010; Yan & Wang, 2006) and more prone to aggregation than $A\beta_{40}$ (Yan & Wang, 2006). $A\beta_{42}$ has specifically been described as the more toxic form of Aβ (Querfurth & Laferla, 2010; reviewed [Tai, Youmans, Jungbauer, Yu, & Ladu, 2011]).

The elevated Aβ levels in the brain drive the conversion of soluble forms of Aβ to aggregated forms of Aβ (Holtzman, 2004), forming amyloid plaques within the extracellular spaces of the brain. Amyloid plaques exist in different conformations. These conformations include diffuse plaques, compact plaques, and neuritic plaques in increasing chronological and neuropathological order (Groen, Liu, Ikonen, & Kadish, 2003). Diffuse plaques, which contain nonfibrillar Aβ, develop from aggregated soluble peptides. Diffuse plaques are displayed as amorphous wisps of amyloid that lack a central core and are probably not neurotoxic. The reason they are important to be studied is because of their progression into neurotoxic plaques. Diffuse plaques have a low β-

pleated sheet content (Holtzman, 2004). Compact "amyloid" plaques are composed of a dense thioflavin-S-positive core, and neuritic plaques are identified as amyloid plaques surrounded by a ring of dystrophic neurites (axons/dendrites; (Groen et al., 2003). Compact and neuritic plaques contain fibrillar Aβ, which possess a high β-pleated sheet structure (Holtzman, 2004; Mattson, 1997).

As described by Games et al. (1995), it is the enhanced production of Aβ peptide (specifically $A\beta_{42}$) that leads to the pathological deposition of amyloid in the brain. It is the accumulation of these Aβ deposits in the brain that leads to the development of the Aβ plaques that eventually lead to neuronal dysfunction in the brain (Koudinov, Berezov, & Koudinova, 2001). Although the mechanism of amyloid neurotoxicity remains unclear (Stern et al., 2004; reviewed [Tai et al., 2011]; Verghese, Castellano, & Holtzman, 2011), research suggests that Aβ has been found in mitochondrial membranes, impairing mitochondrial function (Lustbader et al., 2004; Mattson, Partin, & Begley, 1998; Reddy, 2009). Mitochondria are the major source of energy for normal functioning brain cells. However, evidence linking Aβ toxicity with mitochondrial dysfunction is still limited to *in vitro* studies. Stern et al. (2004) has also suggested that the Aβ plaques disrupt the synchrony of convergent inputs, reducing the ability of neurons to successfully integrate and propagate information. Amyloid deposition in the brain's parenchyma and cerebral blood vessels is one of the recognized neuropathological features of AD. These distinct neuropathological phenotypes will be quantified to assess the degree of Alzheimer's-like pathology in the current study.

NFTs and NTs

Another neuropathological hallmark of AD is intracellular neurofibrillary tangles (NFTs) and neuropil threads (NTs), which consist of abnormal phosphorylated Tau proteins (Richard E Hartman et al., 2005). Tau proteins stabilize microtubules, a component of the cytoskeleton. When tau becomes hyperphosphorylated (multiple phosphates attached to it), tau becomes insoluble and lacks affinity for microtubules. This causes the hyperphosphorylated tau to detach and self-aggregate and microtubules to destabilize, which can lead to the death of the cell.

Cell Loss

Although neuronal loss does not belong to the diagnostic criteria of AD (Duyckaerts, Potier, & Delatour, 2007), it is considered an important pathological component of the disease. Several studies have addressed both the presence and absence of cellular loss in the hippocampus. Hippocampal cell loss was observed *in vitro* in both human and animal models (Estus et al., 1997; Friedrich et al., 2010; Ji et al., 2002; Loo et al., 1993) as well as *in vivo* in mouse models (Calhoun et al., 1998; Casas et al., 2004; LaFerla, Hall, Ngo, & Jay, 1996; Schmitz et al., 2004) in some studies, but not observed in other *in vivo* mouse studies (Irizarry et al., 1997; Jawhar, Trawicka, Jenneckens, Bayer, & Wirths, 2012; Takeuchi et al., 2000). This contradicting evidence suggests that hippocampal cellular loss in AD is something to be further investigated.

Familial vs. Sporadic AD

AD can present as early onset familial AD (FAD) or later onset "sporadic" AD. In

familial AD (FAD), the less common of the two, autosomal genetic mutations directly cause the disease. This form of AD accounts for 1-5% of the total cases of AD (Demattos et al., 2000). Most of these cases occur before the age of 60, which is why it is often called early-onset FAD. FAD is caused by inheriting abnormal variations of the amyloid precursor protein (*APP*) gene, the presenilin 1 (*PSEN1*) gene, and/or the presenilin 2 (*PSEN2*) gene.

In the more common sporadic/late-onset form of AD, genes influence the risk of developing the disease rather than directly causing the disease. Sporadic AD usually occurs after the age of 60, which is why it is often called late-onset sporadic AD. The most-studied gene of susceptibility in sporadic AD is the apolipoprotein E (*APOE*) gene. As shown in Table 1, other genes, including *α2M*, *LRP*, *ACE*, and *VLDL-R,* can also influence susceptibility to sporadic AD (Rocchi, Pellegrini, Siciliano, & Murri, 2003).

Table 1. Genes involved in familial (bold) and sporadic AD.

Gene name	Chromosomal location	Onset	Familial and/or sporadic	Involvement in AD
APP	**21q21.3–q22.05**	**Early**	**F**	**Certain**
PS1	**14q24.3**	**Early**	**F**	**Certain**
PS2	**1q31–q42**	**Early**	**F**	**Certain**
APOE	19q32.2	Late	S and F	Certain
α2M	12p	Late	S	Uncertain
LRP	12	Late	S	Uncertain
LBP-1c/CP2/LSF	12	Late	S	Uncertain
ACE	17q23	Late	S	Uncertain
VLDL-R	9pter–p23	Late	S	Uncertain
BChE	3q26.1–q26.2	Late	S	Uncertain
ACT	14q32.1	Late	S	Uncertain
IDE	10q23–q25	Late/early	S and F (?)	Uncertain
Tf C2	3q21	Late	S	Uncertain
catD	11p15.5	Late/early	S and F	Uncertain
BH	17q11.1–q11.2	Late/early	S	Uncertain
TGF-β1	19q13.1–q13.3	Late	S	Uncertain
5-HTT	17q11.1–q12	Late	S	Uncertain
APOE promoter	19q32.2	Late/early	S	Uncertain
NOS3	7q35	Late	S	Uncertain
CST3	20p11.2	Late	S	Uncertain
PS1 promoter	14q24	Early	S and F	Uncertain

Genetic Risk Factors

APP

The inheritance of mutated *APP* is a risk factor for FAD. Certain mutations in *APP* result in the development of higher than normal levels of Aβ, leading to the accumulation of Aβ plaques. This eventually causes neuronal dysfunction in the brain. The processing of APP to Aβ occurs in normal individuals as well as those with AD, so it is presumed that the development of AD is due to a mutation in this gene, which leads to a change in either the quantity or quality of Aβ (LaFerla et al., 1996). These mutations are a relatively rare cause of AD (Schellenberg, 1995). Since *APP* is located on chromosome 21 and Down syndrome is caused by trisomy 21 (3 copies of chromosome 21), those with Down syndrome are more likely to developing AD.

Presenilin 1 and 2

Once APP mutations were found, extensive research suggested that these mutations accounted for only a small subset of AD cases. Genetic linkage analysis led to the identification of a chromosome 14 early-onset AD locus (Schellenberg, 1995) Presenilins are a family of transmembrane proteins that act as a γ-secretase (an enzyme that cleaves transmembrane proteins at residues within the transmembrane domain). The presenilin 1 (*PSEN1*) gene and presenilin 2 (*PSEN2*) gene have been identified as a cause of FAD (Schellenberg, 1995). Mutations in these genes are thought to cause up to 80% of FAD cases (Rocchi et al., 2003).

APOE

APOE is a gene located on chromosome 19 that has been shown to play a factor in the pathogenesis of late-onset AD. *APOE* has three major alleles: *APOE2* (E2), *APOE3* (E3), and *APOE4* (E4). These alleles produce the isoproteins apolipoprotein E2 (apoE2), apoE3, and apoE4, respectively (Eto et al., 1986). The likelihood of one developing AD is highly dependent upon which of these three alleles they carry (Corder et al., 1993). ApoE is a ligand for the low-density lipoprotein receptor (LDLR), which is involved in maintaining cholesterol and triglyceride homeostasis (Sullivan et al., 1997). A lipoprotein is a group of soluble proteins that transports fats or lipids to the blood plasma. A low-density lipoprotein (LDL) carries cholesterol throughout the body and delivers it to different organs and tissues. LDL is also considered the "bad" cholesterol since it contributes to plaques. High-density lipoprotein (HDL) picks up excess cholesterol in the blood and takes it back to the liver where it is broken down. It is considered as "good" cholesterol since it helps remove LDL cholesterol from the arteries.

After the liver, the brain expresses the highest expression of apoE, particularly in areas involved in neural development, regeneration, and remodeling (Grootendorst et al., 2005; Kim, Basak, & Holtzman, 2009). The liver synthesizes about 70% of apoE in the body, and the brain synthesizes about 20% (Sullivan et al., 1997). Similar levels of apoE mRNA have been found in the cerebellum, medulla, and cerebral cortex of the rat brain (Elshourbagy, Liao, Mahley, & Taylor, 1985). Besides the liver and the brain, apoE is also synthesized in the spleen, lungs, adrenal glands, kidneys, and muscles (Blue, Williams, Zucker, Khan, & Blum, 1983; Tokuda et al., 2000). Major cell types that express apoE in the brain include astrocytes and microglia (Kim et al., 2009). ApoE has

shown to play a role in the brain's response to injury (Poirier, 1996; Tokuda et al., 2000). Following injury, astrocytes and macrophages (cells that function in phagocytosis) synthesize and release apoE within the lesion to repair the nervous system through membrane synthesis (Poirier, 1996; Tokuda et al., 2000).

Corder et al. (1993) studied the genotype of 42 late-onset families that were diagnosed with AD. Each individual has two *APOE* genes on chromosome 19. One copy is inherited from each parent. With increasing numbers of E4 alleles, they found that the risk for AD increased from 20% to 90%, and the mean age of onset decreased from 84 to 68 years. Compared to individuals lacking E4, those with one E4 allele are 2 to 3 times more likely to get AD, and those with two E4 alleles are about 12 times more likely to get AD (Bertram, McQueen, Mullin, Blacker, & Tanzi, 2007). The E4 allele is known to increase the risk and decrease the age of onset of AD, whereas the E2 allele is known to decrease the risk and increase the age of onset of AD. The E4 allele represents a major risk for AD in a variety of ethnic groups (Farrer et al., 1997). A pilot study has found a protective role in the E4 allele in the cognitive development in children with heavy diarrhea in Northeast Brazil (Oriá et al., 2005).

The E3 allele encodes for the most common isoform and acts as a neutral allele, not modifying the risk of developing the disease. From a normal representative United States sample with a similar number of young and elderly males and females (n = 1209; age range 22-71 years), the major *APOE* alleles, E2, E3, and E4, occurred at frequencies of 7.5, 78.6, and 13.5%, respectively (Ordovas et al., 1987). Other rare isoforms (E1, E5, and E7) have been reported (Weisgraber, Rail, Innerarity, & Mahley, 1984; Yamamura, Yamamoto, & Sumiyoshi, 1984). The E1 and E5 alleles were found to occur at

frequencies of 0.2% each (Ordovas et al., 1987). Hallman et al. (1991) found no evidence that the effects of apoE polymorphism on plasma cholesterol levels were significantly different among the nine populations studied; Tyrolean, Sudanese, Indian, Chinese, Japanese, Hungarian, Icelandic, Finnish, and Malay. The frequencies did vary by population though. The Chinese and Japanese appeared to show higher frequencies of ε3 and lower frequencies of ε4 than did the other populations. The high frequency of ε3 in both populations has been reported in previous studies (Asakawa, Takahashi, Rosenblum, & Neel, 1985; Eto et al., 1986; Tsuchiya et al., 1985).

The three alleles differ in their affinity for the LDLR. ApoE3 and apoE4 have a 100% binding affinity to LDLR (leading to an increase in LDL cholesterol), while apoE2 only has 1% normal binding affinity (leading to a decrease in LDL cholesterol; Sullivan et al., 1997). Despite the lowing binding affinity of apoE2 for the LDLR, individuals homozygous for ε2 usually have lower than normal plasma cholesterol levels, except for the fraction of homozygotes (5-10%) who develop type III hyperlipoproteinemia (Utermann, 1982).

Schmechel et al. (1993) selected autopsy-confirmed cases of late-onset AD without affected family members and without other neurological disease as subjects for their study. They found that the average neuritic plaque counts increased with two, as opposed to one, copy of E4, and that the number of neuritic plaques was greater in the E4 than in the E4 homozygotes. To further investigate this trend, Schmechel et al. (1993) looked at the impact that E3 and E4 alleles had on Aβ plaques in brain tissue sections. They reported that homozygotes for E4 had a 5- to 7-fold greater average area covered by strongly Aβ plaques compared to E3 homozygotes. Additionally, they found that there

was a significant dose-related increase of vascular amyloid with the number of E4 alleles. No vascular amyloid was observed in most of E3/3 cases, some vascular amyloid was observed in E3/4 cases, and large amounts were reported for E4/4 cases (Schmechel et al., 1993).

Previous research using *in vitro* models report that E4 enhances Aβ production whereas E3 does so to a lesser extent (Mahley, Weisgraber, & Huang, 2006; Ye et al., 2005). Ye et al. (2005) applies these findings to some *in vivo* findings to explain why E4 is associated with greater Aβ deposition than E3 in AD brains and in transgenic mice expressing mutant APP. Various mouse models suggest how apoE4 can lead to the development of Aβ deposits (Bales et al., 1999; Ye et al., 2005). As described by Games et al. (1995), the development of Aβ plaques is derived from an accumulation of these Aβ deposits, supporting the hypothesis that apoE4 mice will have more AD-like pathology than apoE3 mice.

Others

As mentioned earlier, other genes involved in AD include *α2M*, *LRP*, *ACE*, and *VLDL-R* (Rocchi et al., 2003). A2-Macroglobulin (α2M) is a serine protease inhibitor, or a protein that inhibits the breakdown of other proteins, that is expressed in the brain and found in neuritic plaques (Dodel et al., 2000). The *α2M* gene is located on chromosome 12p. A genetic linkage was detected in late-onset families for a susceptibility gene on chromosome 12 (Pericak-vance et al., 1997), which is why *α2M* became a candidate as a disease locus for FAD. α2M plays a role in mediating the clearance and degradation of Aβ (Blacker et al., 1998). Blacker et al. (1998) reported an association between a deletion

in exon 18 of *α2M* and AD in a sample of affected and unaffected siblings segregating AD. His study confirmed the risk of AD (OR=3.55) comparable to that of ε4 (OR=3.54). However, studies using worldwide populations as samples failed to confirm such an association (Dow et al., 1999; Rogaeva et al., 1999). For that reason, the possibility exists that this *α2M* deletion could be a risk factor for FAD only (Rocchi et al., 2003).

The *LRP* gene (low density lipoprotein receptor-related protein) is another candidate for a potential association with AD. LRP is the main apoE receptor expressed in neurons (Rebeck, Reiter, Strickland, Hyman, & Cross, 1993), is responsible for the endocytosis (the process of cells absorbing molecules by engulfing them) of secreted APP (Kounnas et al., 1995), and is detected in senile plaques (general term for Aβ plaques in the elder population), dystrophic neurites and reactive astrocytes in the AD brain (Rebeck, Harr, Strickland, & Hyman, 1995). Multiple studies have shown that certain polymorphisms in *LRP* may play as a minor risk factor for sporadic AD (Kamboh, Ferrell, & DeKosky, 1998; Kang et al., 1997; Lendon et al., 1997).

The *ACE* gene (angiotensin converting enzyme) encodes for an enzyme that catalyzes the conversion of angiotensin I to angiotensin II (Rocchi et al., 2003), a protein that causes vasoconstriction. A polymorphism in *ACE* has been found to be associated with AD susceptibility (Kehoe et al., 1999). Subsequent studies confirmed that certain polymorphisms in *ACE* lead to an increased risk in late-onset AD (Alvarez et al., 1999) including in Chinese (Cheng, Hong, Liu, Liu, & Tsai, 2002) and Japanese (Hu et al., 1999) populations.

The *VLDL-R* gene (very low density lipoprotein receptor) is located on chromosome 9. *VLDL-R* has shown to be found on microglia, particularly those

associated with senile plaques (Christie, Chung, Rebeck, Strickland, & Hyman, 1996). A number of genetic studies have investigated the effect that a polymorphic trinucleotide (CGG) repeat sequence of *VLDL-R*, with alleles ranging from four to nine repeats, will have on the risk for AD. A study investigating the Japanese population homozygous for the five repeats allele leads to an increased risk for AD (Okuizumi et al., 1995). Contrary to that study, there were no significant differences between AD cases and controls in allele frequencies of the CGG repeat polymorphism in the Caucasian population (Okuizumi et al., 1996). A case study was then done investigating these effects in late-onset AD patients from Northern Ireland (McIlroy et al., 1999). Findings suggested carriers of 9,9 genotype of *VLDL-R* are at increased risk of AD in Northern Ireland. Although the genetic studies from distinct populations on the CGG repeat polymorphism in the 5'-untranslated region of *VLDL-R* obtained inconsistent results, the possibility of *VLDL-R* acting as a susceptibility gene for AD exists.

Animal Models

Natural AD Development

Before the development of molecular genetics, the study of genes and pathology was limited to inherited characteristics or spontaneous mutations. Selective breeding was a common method that was used. Natural AD development has been studied in chickens, dogs, non-human primates, dolphins, bears, guinea pigs, and rabbits because of their highly homologous Aβ sequence to that of humans. Rats and mice, on the other hand, have a distinct Aβ sequence from humans that does not naturally aggregate into a β-sheet conformation. Chickens, dogs, non-human primates, dolphins, and bears have shown age-

related human-like AD neuropathology, whereas, despite their homologous Aβ sequence to that of humans, guinea pigs, and rabbits did not.

Carradeguas et al. (2005) revealed that the sequence of chicken *APP* is very similar to the human sequence of *APP*. They also reported that the chick embryo processes APP by both amyloidogenic (cleavage by β- and γ-secretases, leading to the formation of Aβ plaques) and non-amyloidogenic (cleavage by α-secretase, preventing the formation of Aβ plaques) pathways. In addition to the chicken Aβ sequence's similarity to that of humans, they also reported that $A\beta_{42}$ was the major Aβ peptide produced during chick embryogenesis (the formation and development of an embryo), suggesting that the chick embryo may be appropriate for studying the mechanisms of Aβ production.

Age-related Aβ deposition was reported in the prefrontal, occipital, parietal, and entorhinal cortices of 40 beagle dogs ranging from 2 to 18 years of age (Head, McCleary, Hahn, Milgram, & Cotman, 2000). Head et al. (2000) also found that the earliest and most consistent site of Aβ deposition with age was in the prefrontal cortex, similar to what is seen in the aging human. It has also been reported that the extent of the Aβ is correlated with learning and memory impairments in aging dogs (Cummings, Head, Afagh, Milgram, & Cotman, 1996a; Cummings, Head, Ruehl, Milgram, & Cotman, 1996b).

Natural models of non-human primates, dolphins, and bears have been studied as well (Cork et al., 1988; Sarasa & Pesini, 2009). Unlike wild-type mice, many non-human primates naturally develop Aβ plaques (Lemere et al., 2008). Lemere et al. (2008) found age-related plaque deposition in tamarins older than 12 years of age. Also, tissue samples

from stranded dolphins were found to have extensive positive Aβ-immunolabeled deposits throughout the brain, including the cerebellum and medulla oblongata where such deposits have only been reported in more severe cases of AD (Luo et al., 2001). The $A\beta_{42}$ peptide in three dolphin species was found to be 100% identical to the human peptide (Sarasa & Pesini, 2009). Furthermore, two different types of bears have also shown age-related human-like AD pathology (Cork et al., 1988). A 20-30 year old Asiatic brown bear and polar bear displayed NFTs and numerous senile plaques, respectively (Cork et al., 1988).

Although the Aβ sequence in guinea pigs and rabbits happen to be identical to that of human (Johnstone, Chaney, & Norris, 1991), Sarasa and Pesini (2009) reported that these animals did not present AD pathology spontaneously. Guinea pigs did not develop the pathological features of AD, such as senile plaques and neurofibrillary tangles. Although rabbits did not present AD pathology spontaneously, Wu et al. (2003) reported that increased dietary cholesterol levels in rabbits led to increased Aβ and apoE concentrations in the temporal and frontal cortices, regions where amyloid deposits have been displayed in the pathology of AD.

Perhaps other factors, aside from the similar Aβ sequence to that of humans, may describe the possible mechanism for why guinea pigs and rabbits do not develop age-dependent AD-like neuropathology. The natural development of amyloid deposits may be closely linked with the concentrations of β- and γ- secretase in the brain or a specific diet that these animals generally have.

Non-Transgenic Models

Before transgenic animals models became popular, non-transgenic animal models were used. In these cases, the genetic material of the animal was not altered. Examples consisted of injecting the $A\beta_{40}$ peptide into rat brains via intrahippocampal injection (Stéphan, Laroche, & Davis, 2001) or the $A\beta_{42}$ peptide via intracerebroventricular infusion (Lecanu, Greeson, & Papadopoulos, 2006; Nakamura, Murayama, Noshita, Annoura, & Ohno, 2001). The overall goal in these studies was to inject a form of amyloid peptide into the rat brain so that the animal would develop the pathological features representative of AD. Alternative non-transgenic animal models include streptozotocin injections (Y. Chen et al., 2013), okadaic acid-induced tau protein hyperphosphorylation (Y.-W. Lim et al., 2010), and aluminum salt administration (Aly, Metwally, & Ahmed, 2011). Also, a study from our group, in which a controlled cortical impact (CCI) injury was administered to the parietal cortex of male rats at postnatal day 17, reported higher levels of endogenous rodent Aβ in several brain regions (Pop et al., 2012). These findings suggest that juvenile traumatic brain injury (jTBI) animals may be used an effective non-transgenic model for AD.

Transgenic/Knockout Models

The term "transgenic" refers to the artificial introduction of genetic material from one organism to another. The first genetically modified animal was created in 1974, by Rudolf Jaenisch (Jaenisch & Mintz, 1974). He had inserted a DNA virus into an early-stage mouse embryo and showed that that inserted gene was present in every cell, but the transgene (the gene that has been transferred from one organism to another) was not

passed to their offspring. In 1981, transgenic mouse models had successfully shown transmission of genetic material to subsequent generations (Brinster et al., 1981; Constantini & Lacy, 1981; Gordon & Ruddle, 1981). Gordon and Ruddle (1981) used one of today's most common methods for generating transgenic mice, DNA microinjection. This method consists of injecting a single gene or a combination of genes from one organism to another. This insertion of DNA is a random process, and there is no way to ensure that the DNA will insert itself into a site on the host DNA that will permit its expression.

Another method of generating transgenic mice is by way of embryonic stem cell-mediated gene transfer. The first recorded KO mouse was in 1989 (Capecchi, 1989). This method consists of inserting mutant embryonic stem (ES) cells, which are undifferentiated cells that still have the potential to differentiate into any type of cell (somatic and germ cells), into blastocysts in an embryo. This allows for the insertion of a certain gene in a specific location of the researcher's choice.

APP Models

Mutations

Prior to the development of PDAPP mice, animals transgenic for APP had failed to show extensive AD-like neuropathology (Lamb et al., 1993; Quon et al., 1991). Quon et al. (1991) generated transgenic mice that expressed APP-751, an isoform of APP. These mice exhibited poorly resolved extracellular Aβ deposits in the hippocampus and cortex. Lamb et al. (1993) introduced *APP* into mouse embryonic stem cells, which showed no evidence of AD-type pathology.

Once FAD-associated mutations in APP had been discovered, the focus of making AD transgenic mouse models turned to including the overexpression of transgenes containing FAD mutations (Elder et al., 2010). Games et al. (1995) created the first successful AD transgenic mouse model that contained an FAD-associated mutation. The resulting high level of human mutant APP in the brains of these mice resembled that of early-onset FAD. This model consists of a human *APP* minigene, or a segment of *APP* that codes for a portion of the APP protein, with a mutation that causes FAD (APP^{V717F}). The nomenclature of the mutation suggests that the valine amino acid (V) gets replaced with the phenylalanine amino acid (F) at residue 717 along the APP peptide. The FAD mutation at residue 717 shifts the production of Aβ from the 40-amino-acid form to the longer and more amyloidogenic 42-residue peptide known to predominate in plaques (a. E. Roher et al., 1996; Suzuki et al., 1994). It is also highly expressed in the central nervous system (Elder et al., 2010). This line became known as PDAPP (Platelet-Derived growth factor promoter driving Amyloid Precursor Protein; Elder et al., 2010). In the PDAPP transgenic mouse model, mice express high levels of human mutant APP, causing neuropathology resembling that of early-onset FAD. This causes the mice to progressively develop many of the pathological hallmarks of AD (Games et al., 1995), such as age-dependent amyloid deposition in the brain, along with thioflavin-S-positive plaques (Elder et al., 2010). PDAPP mice also developed age-related learning defects (Richard E Hartman et al., 2005) and synapse loss (Elder et al., 2010). The first PDAPP line generated showed no obvious pathology before about 6 months of age (Games et al., 1995). At about 6-9 months of age, these transgenic mice showed Aβ deposits in the hippocampus, corpus callosum, and cerebral cortex.

Another line of transgenic mouse created from FAD-associated mutations is the Tg2576 mouse (Elder et al., 2010). These mice, also known as APPsw, overexpress a human APP transgene containing the Swedish FAD mutation (K670N/M67IL). This is a double mutation where lysine (K) is replaced with asparagine (N) at residue 670, and methionine (M) is replaced with leucine (L) at residue 671. These mice exhibited learning and memory impairments by 9 to 10 months of age with a resulting a five-fold increase in $A\beta_{40}$ and a 14-fold increase in $A\beta_{42}$ accompanying these behavioral deficits (Hsiao et al., 1996). Considering how $A\beta_{42}$ aggregates more rapidly and is more toxic to the neuron compared to $A\beta_{40}$ (Yan & Wang, 2006), these findings suggest the harmful effects of this mutation. PDAPP mice (Fryer et al., 2003) generally have a lower ratio of $A\beta_{40}$:$A\beta_{42}$ compared to Tg2576 mice (Hsiao et al., 1996; Johnson-Wood et al., 1997). Other transgenic AD mouse lines with FAD-type mutations include APP23, TgCRND8, APP-Dutch, PS1M146V, PSAPP, and 3xTg (Elder et al., 2010; Table 2).

Table 2. Selected examples of AD mouse models.

Line	Promoter	FAD Mutation	Amyloid Pathology	Reference
PDAPP	PDGF	APP-Indiana	Parenchymal plaques at 6–9 months of age	12
Tg2576	PrP	APP-Swedish	Parenchymal plaques by 11–13 months of age with some vascular amyloid	16
APP23	Thy-1	APP-Swedish	Parenchymal plaques by 6 months of age and prominent vascular deposition of amyloid	18
TgCRND8	PrP	APP-Swedish + Indiana	More aggressive parenchymal plaque pathology present by 3 months of age	17
APP-Dutch	Thy-1	APPE693Q associated with hereditary cerebral hemorrhage with Dutch-type amyloidosis	Vascular deposition of amyloid with few parenchymal plaques	47
PS1M146V	PDGF	PS1M146V	Elevated Aβ42 without plaque pathology	24
PSAPP	PS1M146V × Tg2576	PS1M146V + APP-Swedish	Earlier and more extensive plaque pathology in comparison with Tg2576 alone	30
3×Tg	Thy-1.2 and native mouse	Transgenes containing Thy-1.2–driven APP-Swedish and tau P301L were coinjected onto a homozygous PS1M146V knock-in background.	Parenchymal plaques by 6 months of age combined with tau pathology by 12 months of age	63

NOTE: Additional information on AD Tg models can be found at the Web site of the Alzheimer's Association (http://www.alzforum.org/res/com/tra).

Abbreviations: Aβ, β-amyloid; AD, Alzheimer's disease; APP, amyloid precursor protein; FAD, familial Alzheimer's disease; PDAPP, platelet-derived growth factor promoter driving amyloid precursor protein; PDGF, platelet-derived growth factor β; PrP, prion protein; PS1, presenilin 1; PSAPP, presenilin/amyloid precursor protein; Tg, transgenic.

APOE Models

Knockout

KO mice are defined as mice whose DNA has been genetically engineered so that it does not express particular proteins. In the current study, that particular protein is apoE. ApoE KO mouse models have been used to investigate the role of apoE in the nervous system. Fagan et al. (1998) found no significant defects in the septo-hippocampal cholinergic system (plays a role in spatial working memory) in aged apoE KO mice (up to 24 months of age). Hartman et al. (2001) found that mice lacking apoE were significantly more active than wild-type mice. Also, Shibata et al. (2000) found that, compared to adult wild-type mice, Aβ clearance was significantly reduced in young and old apoE KO mice.

Human APOE

Other studies investigated the role of human *APOE* in the AD brain by using human apoE KI mouse models. Sullivan et al. (1997) used a gene replacement strategy to generate mice that express human E3 in place of murine *APOE*. Although murine *APOE* is about 70% identical to human *APOE* at the amino acid level, there are inherent functional differences between murine and human *APOE* isoforms on plasma lipoprotein metabolism in vivo (Sullivan et al., 1997). He found that after the 3/3 (E3 KI) and WT mice were fed a high fat/high cholesterol diet, the 3/3 mice responded with an increase in cholesterol 5 times to that of the WT mice. After 12 weeks of being on the diet, the 3/3 mice also developed 13-fold larger atherosclerotic plaques in the aortic sinus area than the WT mice. In contrast to murine *APOE*, human *APOE* isoforms have been known to suppress early Aβ deposition and Aβ levels in 9-month-old PDAPP mice (D M Holtzman et al., 2000; David M Holtzman et al., 1999).

Yao et al. (2004) compared Aβ accumulation among human E3 KI, human E4 KI, and murine *APOE* KO mice. They found that E4 carriers had higher levels of Aβ than either E3 or *APOE* KO mice. Aged male E4 mice appeared to have the highest average values for $A\beta_{40}$, $A\beta_{42}$, and total Aβ (Yao et al., 2004). Grootendorst et al. (2005) was the first study to investigate and compare the behavioral phenotype of KI human E3 and E4. He found that spatial memory deficits occurred particularly in human E4 mice, especially female.

APP x APOE Models

Once *APOE* KO mouse models had led to a more clear understanding of the apoE

protein, *APOE* KO mice were crossed with PDAPP mice. Bales et al. (1999) found that the amount of Aβ immunoreactivity in the hippocampus of transgenic mice overexpressing the V717F human APP with no, one, or two *APOE* alleles was dramatically reduced in an *APOE* dose-dependent manner ($APOE^{+/+} > APOE^{+/-} >> APOE^{-/-}$). Bales et al. (2009) further investigated the role of human *APOE* on Aβ deposition by crossing PDAPP mice lacking murine *APOE* to targeted replacement mice expressing human E4 (PDAPP/TRE4). They found that the brain levels of $A\beta_{42}$ in PDAPP/TRE4 mice were significantly elevated in young mice.

Hartman et al. (2002) investigated these effects in a PDAPP x *APOE* model having undergone traumatic brain injury (TBI). They found that TBI accelerated Aβ deposition, such that PDAPP mice expressing E4 displayed Aβ deposition by 12-13 months of age in the presence of TBI compared to about 15 months of age in the absence of TBI. They also found a greater and earlier amyloid deposition in PDAPP:E4 mice than in PDAPP:E3 mice in the presence of TBI.

Humans with AD

Studies on humans with AD have assessed the differences in neuropathological and behavioral deficits between those who are carriers of the E4 allele versus those who are carriers of the E3 allele. In a study assessing 194 AD cases and 3,984 cognitively normal controls, findings indicated E4 was significantly associated with an increased risk of AD compared to E3 (Lindsay, J., Laurin, D., Verreault, R., Hebert, R., Helliwell, B., Hill, G. B., McDowell, 2002). Specifically, those who had at least one E4 allele had 3.28 times the risk of AD compared to those who were homozygous for the E3 allele.

In terms of neuropathological deficits, the apoE isoform 4, which is the protein encoded by the E4 allele, has a higher avidity in vitro for β-amyloid than the apoE isoform 3 (Corder et al., 1993). This study found that E4 increases the rate and extent of amyloid deposition compared to the E3 allele. Another study (Schmechel et al., 1993), found that late-onset AD patients with one or two E4 alleles have a distinct neuropathological phenotype compared with patients homozygous for the E3 allele. Specifically, AD patients homozygous for the E4 allele had abundant immunoreactive plaques whereas AD patients homozygous for the E3 allele only had slight immunoreactive plaques. These differences were so evident that they were often observed without a microscope. AD patients homozygous for the E4 allele also had more neuritic plaques and NFTs in the CA1 subfield of the hippocampus and cortical regions compared to those homozygous for the E3 allele. In another study, E4:E3 AD patients also had lower cortical thickness values compared to those homozygous for the E3 allele in regions associated with early pathological changes in AD (CA1 subfield of the hippocampus and parietal regions).

In terms of behavioral changes, Bookheimer et al. 2000 found that AD patients who were carriers of the E4 allele had lower scores on a delayed-recall test, although not significantly lower, than did carriers of the E3 allele. They also found significant increases in the functional magnetic resonance imaging (fMRI) signal intensity during learning and recall periods as compared with resting periods. This intensity was greater among the carriers of the E4 allele compared to carriers of the E3 allele, which was later correlated with degree of decline in memory in a longitudinal assessment after two years.

These findings suggested that subjects at genetic risk for AD used greater cognitive effort to achieve the same level of performance as subjects who were not at genetic risk.

Thesis

The double-transgenic mice used for the current study were obtained from the Eli Lilly Corporation in 2009, where they crossbred PDAPP transgenic mice with apoE KO mice. This KO technology consisted of removing the murine *APOE* from their genomes and, via KI technology, replacing it with a human variation of E3 or E4 in the precise location within the mouse genome that the murine *APOE* was removed from. Given the similarities in behavior and neuropathology between humans with AD and transgenic mouse models for AD, transgenic mouse models for AD have frequently been used to gain a better understanding of the disease and to characterize the neuropathological mechanisms.

Hypothesis One

The first hypothesis states that PDAPP:E4 mice will show more Alzheimer's-like pathology in the hippocampus and dorsal cortex than the PDAPP:E3 mice. The hippocampus and cingulate cortex showed dramatic age-related increase in Aβ levels in PDAPP mice (Johnson-Wood et al., 1997) and in mice that co-express five FAD mutations (Oakley et al., 2006). The hippocampus also showed significantly more Aβ load in old PDAPP mice compared to young or middle-aged PDAPP mice (Games et al., 1995) and in PDAPP mice of all ages compared to controls (Demattos et al., 2000).

A study was done to assess the amount of amyloid deposition, which is a main component of Aβ plaques, in three different mouse genotypes of one particular mouse model overexpressing *APP*^{V717F} (PDAPP; Bales et al., 1999). Mice that were homozygous (carrying two identical alleles of a particular gene) for the *APP*^{V717F} transgene were crossed to an *APOE* KO mouse. The resulting hemizygous progeny were subsequently bred, and the following genotypes were selected for the study: *APP*$^{V717F+/-}$:*APOE*$^{-/-}$ (1 copy of the human *APP*^{V717F}; 0 copies of murine *APOE*), *APP*$^{V717F+/-}$:*APOE*$^{+/-}$ (1 copy of the human *APP*^{V717F}; 1 copy of murine *APOE*), and *APP*$^{V717F+/-}$:*APOE*$^{+/+}$(1 copy of the human *APP*^{V717F}; 2 copies of murine *APOE*). The results suggested that in the case of the *APP*$^{V717F+/-}$ *APOE*$^{-/-}$ mice, where the mice were *APOE*-deficient, no amyloid deposits were formed at 9, 15, or 21-22 months. When mouse *APOE* was present (as with the *APP*$^{V717F+/-}$:*APOE*$^{+/-}$ and *AP*P$^{V717F+/-}$:*APOE*$^{+/+}$ mice), the researchers found an age-related increase of amyloid deposits in the hippocampus and the cortex. These results suggest that amyloid deposits develop in cases where *APOE* is present, but not when *APOE* is absent. Although these mice expressed mouse (murine) *APOE* rather than human *APOE*, the findings emphasize the critical role that *APOE* plays in amyloid deposition, especially when there is an overexpression of mutant APP.

Johnson-Wood et al. (1997) measured the total Aβ and APP measurements in PDAPP mice from 4 to 18 months of age. A total Aβ sandwich ELISA (enzyme-linked immunosorbent assay), a test using antibodies and color change to detect a sample antigen by "sandwiching" the antigen in between the two antibodies, was conducted. This test used antibodies 266 and 3D6, which are specific to certain amino acid epitopes

within the Aβ peptide. The Aβ levels appeared to increase significantly by age in the hippocampus (Figure 2).

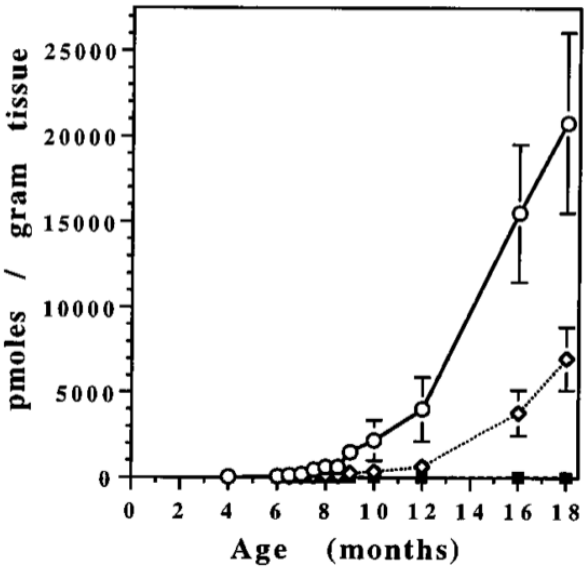

Figure 2. The age-dependent changes in brain Aβ levels in the PDAPP transgenic mice in the hippocampus (white circle), cortex (white diamond), and cerebellum (black square). PDAPP mice were sacrificed at the ages indicated. The Aβ levels increased significantly by age in the hippocampus compared to the other cortex and cerebellum (Johnson-Wood et al., 1997).

There was also an age-related increase in Aβ in the hippocampus of PDAPP mice due to $A\beta_{42}$. $A\beta_{42}$ comprised of 27% of the 17 pmol/g of Aβ present in the brains of young animals and increased to 89% of the 694 pmol/g in the 12-month-old animals. Between 12 and 16 months of age, an even more dramatic increase in deposition of Aβ occurred in the hippocampus and frontal region of the cortex. By 18 months, $A\beta_{42}$ levels in the PDAPP mice were comparable to the higher Aβ levels observed in humans with AD (Gravina et al., 1995). Initially, before amyloidotic deposition, there were 38.1 pmol/g of Aβ in the hippocampus of these young animals. The model used by Johnson-Wood et al.

(1997) is an accurate representation of human AD pathology such that the majority of depositing Aβs in these mice were in the longer $A\beta_{42}$ form, and studies of human AD have shown that the predominant and initially depositing Aβs are the longer form.

APP/PS1 double transgenic mice overproduce $A\beta_{42}$ and exhibit plaque pathology, but transgenic mice develop plaques slowly. The majority of AD transgenic mouse models take about 6-12 months, or longer, to form amyloid plaques (Oakley et al., 2006). To accelerate plaque development, Oakley et al. (2006) investigated APP/PS1 double transgenic mice that co-express five FAD mutations (5XFAD mice) to investigate the effects of high $A\beta_{42}$ levels. The five FAD mutations were APP K670N/M671L (Tg2576 mice previously described), APP I716V (isoleucine is replaced with valine at residue 716 on the APP peptide), APP V717I (valine is replaced with isoleucine at residue 717 on the APP peptide), PS1 M146L (methionine is replaced with leucine at residue 146 on the PS1 peptide), and PS1 L286V (leucine is replaced with valine at residue 286 on the PS1 peptide). These mutations were introduced into the cDNA (complimentary DNA) by site-directed mutagenesis. They found that intraneuronal $A\beta_{42}$ accumulated at 1.5 months of age, before the first appearance of Aβ deposits at 2 months. There was a rapid increase of cerebral $A\beta_{42}$ levels in 5XFAD mice (Oakley et al., 2006). At young ages, these mice generated $A\beta_{42}$ levels almost exclusively (Figure 3). $A\beta_{40}$ levels began to rise at older ages, although always lower than $A\beta_{42}$ levels (Figure 3).

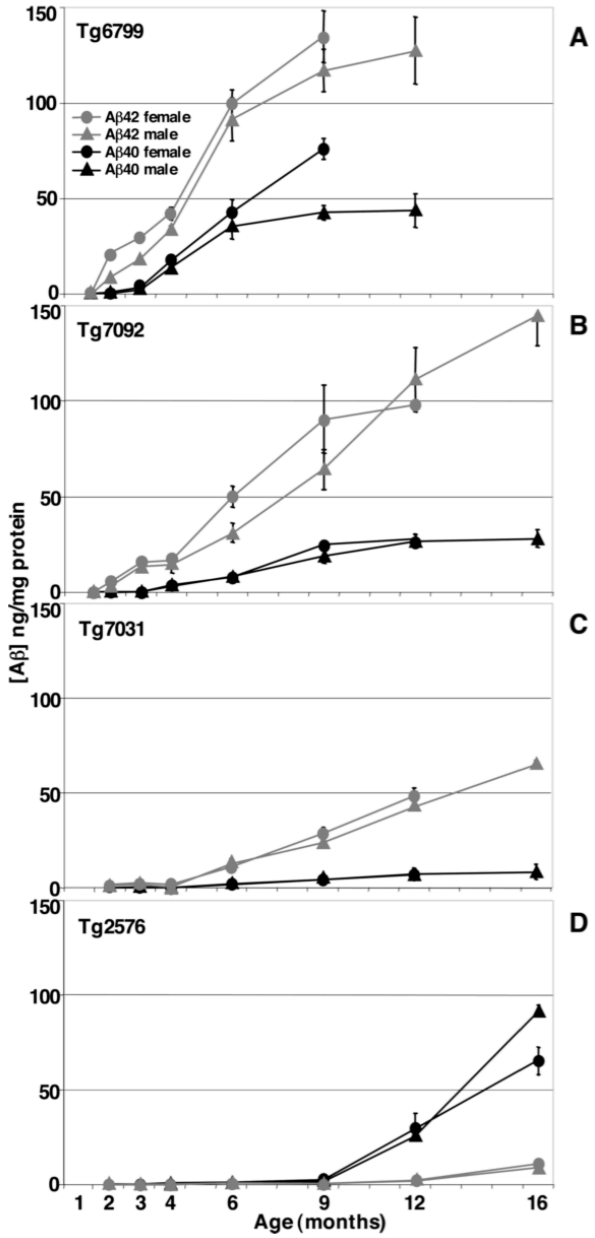

Figure 3. The rapid increase of cerebral $A\beta_{42}$ levels in 5XFAD lines from 1.5 to 16-month-old 5XFAD mice (**A.** Tg6799; **B.** Tg7092; **C.** Tg7031; **D.** Tg2576). (Oakley et al., 2006).

In a study of confirmed cases of AD, in which autopsies were performed within 8 hours from death, those who were homozygous for the E4 allele had increased neuritic plaques compared with patients with no E4 alleles. This suggests a positive correlation

between plaque density and E4 allele dose (Tiraboschi et al., 2004). It is believed that the physical interaction of *APOE* with Aβ plays an important role in AD pathogenesis.

It is the specific effect that E3 and -4 have on the human Alzheimer's brain that are of interest in the current study. The binding affinity of E4 with Aβ is high and has significantly different binding characteristics compared to that of E3 (Schmechel et al., 1993). Schmechel et al. (1993) looked at the relationship that E3 and -4, the two most common alleles of the *APOE* gene, have on Aβ deposition in late-onset AD, since E4 is a susceptibility gene for late-onset AD. They found that patients with sporadic late-onset AD who have one or two copies of E4 have a distinctive neuropathological phenotype of greatly increased vascular and plaque amyloid deposits compared to patients homozygous for E3.

As mentioned earlier, in selected autopsy-confirmed cases of late-onset AD, Schmechel et al. (1993) found more Alzheimer's-like pathology (neuritic plaques, Aβ plaques, and vascular amyloid) in E4 homozygotes than E3 homozygotes. Several other studies, as mentioned earlier, have reported that E4 enhances Aβ production while E3 does so to a lesser extent (Kelly R. Bales et al., 1999; Mahley et al., 2006; Ye et al., 2005). In one mouse model, highly relevant to the current study, Bales et al. (2009) measured the levels of *APOE* protein and Aβ peptides in mice at different ages. The mouse model consisted of crossing PDAPP mice lacking mouse *APOE* to targeted replacement mice expressing human *APOE*. Findings suggested that there was an age-dependent increase in both $A\beta_{40}$ and $A\beta_{42}$, regardless of *APOE* isoform analyzed, which was more pronounced in the PDAPP/TRE4 mice. This study also did not administer any behavioral tests on their mice, something that our study will be addressing. By running

behavioral tests in our study, we are able to analyze any correlates between the behavioral deficits and neuropathology seen in their brains. Having a clear understanding of the impact that specific isoforms of *APOE* play in the development of AD can lead to the development of preventive techniques as well as accurate prognoses of AD patients.

Hypothesis Two

The second hypothesis states that the PDAPP:E4 mice will have fewer cells in the hippocampus and cortex than the PDAPP:E3 mice. The hippocampus, specifically the CA1 hippocampal subfield (Figure 4), is known to have substantial neuronal loss in AD patients (Mann, 1996; West, Coleman, Flood, & Troncoso, 1994) as well as in APP23 transgenic mice (Calhoun et al., 1998).

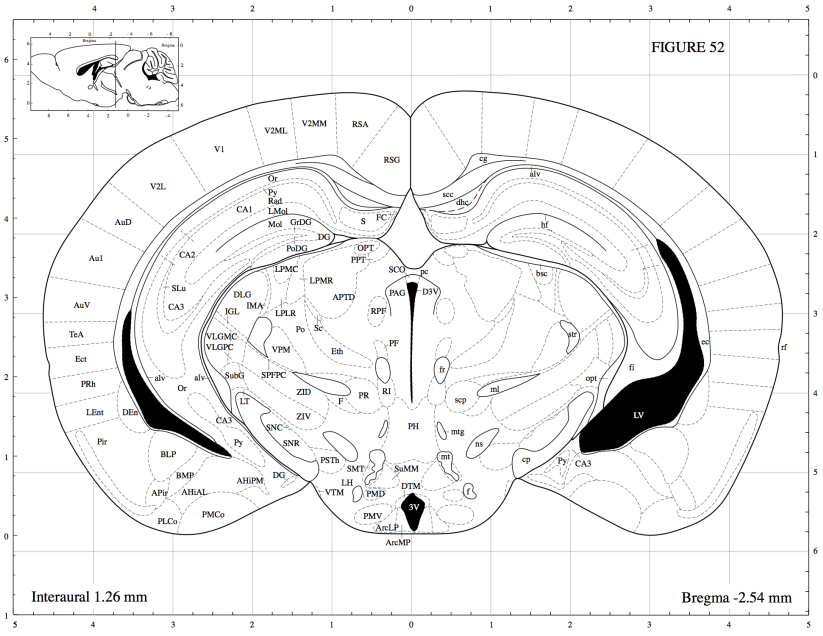

Figure 4. A sagittal view of a wild-type mouse brain. The CA1 hippocampal subfield is known to have substantial neuronal loss in AD patients (Mann, 1996; West et al., 1994) as well as in APP23 transgenic mice (Calhoun et al., 1998).

Several studies of various transgenic mouse models of AD have also reported a lack of cell loss in the CA1 hippocampal subfield (Irizarry et al., 1997; Jawhar et al., 2012; Takeuchi et al., 2000). Although studies have shown both the presence and absence of cell loss in various transgenic mouse strains, the phenomenon has yet to be studied in PDAPP mice that also express human E3 or E4.

In one study (Friedrich et al., 2010), Aβ aggregation was induced *in vitro* by adding dissolved $A\beta_{40}$ to cultures of several cell types (human embryonic kidney cells, neuroblastoma cells, laryngeal carcinoma cells, monocytic cells, simian kidney cells, and a murine macrophage line). These cells lines are relevant to AD in that they all share the ability to enable Aβ plaque formation. They found that plaque biogenesis involves Aβ accumulation within intracellular vesicles, and the fibrils formed by Aβ in these conditions impair the ordered vesicular formation by growing out and penetrating the vesicular membrane. These events lead to the death of the affected cells and the extracellular accumulation of previously intracellular amyloid structures (Friedrich et al., 2010). The cell death reported in this cell-culture system is associated with plaque biogenesis, not necessarily with the massive neuronal loss in AD patients. Duyckaerts et al. (2007) found that transgenic mouse brains can have a lot of Aβ plaque despite very little neuronal death, supporting the notion that cell death is not related to the massive neuronal loss in AD patients.

In another *in vitro* study, the isoform-specific effects of *APOE* on the response of Neuro-2a cells to the Aβ peptides were assessed. Neuro-2a is a mouse neural crest-derived cell line, which is beneficial in the fact that they differentiate into neurons within a few days. This allows for an efficient way of studying neurons. ApoE3- and E4-

transfected Neuro-2a cells were incubated with 20 μM $A\beta_{42}$ for 18 hours at 37ºC, and cell survival was assessed. Transfection is a method of introducing nucleic acids into cells. Findings suggest that $A\beta_{42}$ caused significantly greater cell death in E4-secreting than in E3-secreting or control cells (Ji et al., 2002). This provides evidence for the second hypothesis that E4 leads to more cell death than E3 in the presence of Aβ.

Estus et al. (1997) found that treatment of cultured rat cortical neurons with $A\beta_{40}$ results in a widespread of apoptotic neuronal death. Apoptosis refers to a regulated self-destruction of cells whereas necrosis refers to cell death due to disease, injury, or failure of the blood supply. Finally, Loo et al. (1993) studied cultured cortical and hippocampal neurons from mouse embryos treated with $A\beta_{42}$. They reported that these neurons exhibited classical morphological and biochemical characteristics of apoptosis, which they believe may play a role in the neuronal loss associated with AD. The concentration Aβs used in these *in vitro* studies ranged from 5-40 μM.

In an *in vivo* study, LaFerla et al. (1996) found that their transgenic mouse model (overexpression of the murine homologue of the Aβ peptide), consisting of intracellular expression of Aβ specifically in neurons, led to the death of neurons as well as the functional impairment of neighboring neurons. They found that the hippocampus of the Aβ transgenic mice, among other areas in the cerebral cortex, was an area of apoptotic cells. The observation that Aβ expression induces cell death through the apoptotic pathway *in vivo* may prove relevant for therapeutic approaches to the treatment of AD.

Further transgenic studies have addressed the cell loss associated with APP and PS mutations. In Schmitz et al. (2004), neuronal loss was observed in transgenic mice expressing human mutant APP (APP751) and human mutant presenilin-1 (PS-1 M146L).

This specific mutation in APP consists of the KM670/671NL and V717I mutations. They found no hippocampal granule cell loss (cells located in the dentate gyrus of the hippocampus) at 17 months of age in the APP/PS-1 double transgenic mice as compared to age-matched PS-1 single-transgenic mice and wild-type controls. The neuron loss was observed at sites of Aβ aggregation. The amount of extracellular Aβ aggregates in these APP/PS-1 double transgenic mice increased as they aged, and they found a clear age-related loss of neurons in the pyramidal cell layer of the hippocampus (Schmitz et al., 2004). Specifically, they found a substantial loss of pyramidal neurons in the hippocampus of 17-month-old APP/PS-1 double transgenic mice compared to 4.5-month-old APP/PS-1 double transgenic mice and wild-type controls. The neuronal loss was also observed in areas of the parenchyma, the functional tissue in the brain, distant from plaques. Also, they found no correlation between the number of pyramidal cells and plaque load, which fell in line with a study reporting little or no correlation between the level of neuron loss and the amount of extracellular Aβ in human AD (Gomez-isla et al., 1997). This suggests the possible involvement of more than one mechanism of hippocampal neuron loss in this double-transgenic mouse model of AD. Perhaps part of the hippocampal neuron loss may be due to the high levels of intra-neuronal Aβ independent of extracellular Aβ aggregates.

Another study had reported significant neuron loss in the hippocampal CA1 region in APP23 transgenic mice compared with controls, which was correlated with CA1 plaque load in the transgenic mice within the hippocampus (Calhoun et al., 1998). In this study, the CA1 neuron loss in the APP23 transgenic mice was 14% and had reached 25% in mice with high plaque load (Calhoun et al., 1998). Other studies have

failed to find CA1 hippocampal neuron loss in PDAPP transgenic mice through 18 months of age (Irizarry et al., 1997), PSAPP mice doubly expressing AD-associated mutant presenilin-1 (PS-1) and APPsw (Takeuchi et al., 2000), and 5XFAD mice (Jawhar et al., 2012). Jawhar et al. (2012) found significant pyramidal neuron loss in layer 5 of the cortex, leaving the hippocampus of the 5XFAD mice completely unaffected (Oakley et al., 2006).

A more severe hippocampal neuronal loss is seen a APP/PS1K1 mouse model (Casas et al., 2004). The transgenic mouse model carries M233T/L235P (a double mutation where methionine is replaced with threonine at residue 233 and leucine is replaced with proline at residue 235) KI mutations in presenilin-1 and overexpresses mutated human APP. At 10 months of age, these transgenic mice showed an extensive neuron loss (>50%) in the hippocampus. The neuron loss was correlated with the accumulation of intraneuronal Aβ and thioflavin-S-positive intracellular material.

As addressed by our first hypothesis, E4 leads to greater Aβ production than does E3 in transgenic mice and humans (Kelly R. Bales et al., 1999, 2009; Mahley et al., 2006; Schmechel et al., 1993; Tiraboschi et al., 2004; Ye et al., 2005). Previous studies have also shown that Aβ aggregation induces neuronal cell death (Estus et al., 1997; LaFerla et al., 1996; Loo et al., 1993). Intuitively, one would induce that E4 must lead to more neuronal cellular death than E3, but research has shown no correlation between Aβ deposition and neuronal loss (Gomez-isla et al., 1997; Schmitz et al., 2004). Although, the pyramidal neuron loss in cortical layer 5 (Oakley et al., 2006) occurred in brain regions with the highest levels of both intraneuronal Aβ accumulation and amyloid plaque burden. In fact, the actual link between amyloid plaques and neuronal loss has yet

to be established (Schmitz et al., 2004). The current study investigates neuronal loss in the PDAPP x apoe3 vs. apoe4 model.

Hypothesis Three

The third hypothesis states that the Alzheimer's-like pathology will be positively correlated with the behavioral deficits displayed in the mice. In humans with AD, cognitive and behavioral disturbances caused by the disease do not become evident until years after significant neuropathology, which includes a large amount of plaques accumulating and the occurrence of cell death (Morris & Price, 2001). Hartman et al. (2005) reported that PDAPP mice had substantial learning impairments from early ages, as well as an age-dependent decline in learning ability. Whereas both non-transgenic and PDAPP mice showed evidence of decreased performance with age in the spatial learning task, the performance of PDAPP mice declined much more severely. This suggests that age-related Aβ buildup may have negative consequences for spatial learning. They then treated the non-transgenic and PDAPP mice with anti-Aβ antibody 10D5, a reagent specific for human Aβ, to determine whether this learning impairment was due to Aβ accumulation. They found that aged PDAPP mice exhibited significant improvements in the spatial learning task, suggesting that Aβ contributed to the age-dependent learning deficits displayed in PDAPP mice, providing support for our third hypothesis. Similarly, in another study in which spatial learning performance was tested in PDAPP and non-transgenic mice, a significant negative correlation between spatial learning capacity and plaque burden existed for middle-aged and old PDAPP mice (G. Chen et al., 2000). They

also found that PDAPP mice had a significant age-related increase of plaque burden in the hippocampus.

The overall goal of the current study is to determine the roles that E3 and -4 play in a PDAPP x *APOE* double transgenic mouse model. Understanding this role will provide further insights into its pathophysiological role in AD. Also, these further insights will lead to the development of more effective treatments and therapeutics for the disease.

Successful Completion of Aims

Achievement of the first two aims will allow us to determine the effects of apoE isoforms and overexpression of human mutant APP on AD-like pathology and cell count within the hippocampus and dorsal cortex of transgenic mice that express human mutant APP and human *APOE*. The third aim will allow us to determine the effects of the AD-like pathology, specifically Aβ plaques, on behavior, suggesting that reduction of Aβ could be of potential therapeutic benefit for AD. By understanding where the plaque deposition and cell loss is located, therapeutic benefits may target cognitive functioning associated with those regions of the brain.

Achievement of these three aims will aid in a more formal understanding of the precursors to this disease, specifically *APP* and *APOE*. This will give incentive in developing more effective primary treatment and preventative interventions for AD. With effective primary treatment developed, there will be more motivation for individuals to get tested for their *APP* and *APOE* genes, allowing for an earlier detection of AD. This will likely decrease the prevalence of AD. Determining whether Alzheimer's pathology is

correlated with behavioral deficits will improve technologies as well. Aside from developing effective treatment, technologies will be developed to make living with AD more manageable. By accepting the fact that behavioral deficits are inevitable, scientists and researchers may develop effective computer programs that make those behavioral deficits manageable in their day-to-day routine.

If the first and second hypotheses are supported, then the detrimental effects of E4 on the human brain may be accurately represented in this double transgenic KI mouse model. Since those who are homozygous for E4 do not invariably develop AD, the exact mechanism for how *APOE* alleles influence the risk of AD is still unclear (Kelly R. Bales et al., 2009; Corder et al., 1993; Kim et al., 2009; Mahley et al., 2006). According to Kim et al. (2009), strong evidence suggests that *APOE* influences the risk of AD via its effects on Aβ metabolism. ApoE gets lipidated by ABCA1 to form lipoprotein particles. ABCA1 (ATP-binding cassette A1) transfers cellular cholesterol and phospholipids onto lipid-poor apolipoproteins to form pre-HDL. This lipidated apoE binds to soluble Aβ, which influences the formation of parenchymal amyloid plaques and transport of Aβ within the CNS. Hence, increasing the lipidation state of apoE may influence the Aβ metabolism in the mouse brain or the ability of Aβ to form amyloid fibrils. It is believed that *ABCA1* deletion increases Aβ deposition by affecting the lipidation state of apoE (Wahrle et al., 2005). However, the details of this mechanism are not clearly understood (Kim et al., 2009).

Strong evidence suggests that a major mechanism underlying the link between apoE and AD is related to the interaction between apoE and Aβ and apoE's ability to influence Aβ's clearance, aggregation, and conformation (D. Holtzman, 2004; Tokuda et

al., 2000). Different apoE isoforms alter AD pathogenesis via their interactions with Aβ peptide (Wahrle et al., 2005). The mechanism by which apoE interacts with Aβ is still not clearly understood, but murine apoE has been found to somehow influence Aβ to a β-pleated sheet conformation (K. R. Bales et al., 1997; Kelly R. Bales et al., 1999). Holtzman (2004) investigated the effects of human apoE isoforms on Aβ deposition *in vivo*. Holtzman (2004) found that although PDAPP$^{+/-}$ mice in the presence or absence of murine apoE developed Aβ deposits by 9 months of age, PDAPP$^{+/-}$ mice with human E2, 3, and 4 did not develop Aβ deposition until 15-24 months of age. At 15 months of age, the E3 and 4 mice developed Aβ deposition in the form of both diffuse and neuritic plaques, similar to that seen in the presence of murine apoE. By 18-22 months of age, some E2 mice had begun to develop diffuse plaques, but none had developed neuritic plaques. This data suggests the isoform-specific role in the apoE-Aβ interaction.

CHAPTER THREE

INNOVATION

The specific animal model used in the current study is distinct from previous studies investigating PDAPP mice expressing human E3 and E4 in that they did not assess behavior (Kelly R. Bales et al., 1999; Fagan et al., 2002; D M Holtzman et al., 2000; David M Holtzman et al., 1999). Hartman et al. (2002) investigated PDAPP mice expressing human E3 and E4 as well, with the addition of TBI. In analyzing the neuropathology of AD in mouse brains, the current study addresses a novel way of producing the double-transgenic mice, representing a "cleaner" double-transgenic mouse model compared to what is normally seen in previous literature. In the current study, *APOE* KO mice had human variations of E3 and E4 replaced via KI technology. KO mice are mice whose DNA has been genetically engineered so it does not express a certain protein. These human variations of *APOE* were placed in the exact location within the mouse genome that the mouse *APOE* was removed from. Hypothetically, this resulted in an Aβ and *APOE* interaction more similar to what is seen in humans with AD. These mice were then crossbred with PDAPP transgenic mice. The advantage to using this specific double-transgenic mouse model is that it leads to a more accurate representation of the human Alzheimer's brain.

Additionally, this current study measures the cognitive and behavioral deficits resulting from this specific double-transgenic mouse model. Behavioral deficits have been investigated in the past, but of transgenic animal models that were different from what was used in the current study. Bales et al. (2009) had performed a study using a similar mouse model to ours, but did not study behavior. The current study will not only

assess the neuropathology of the disease brought about by the interaction of human *APOE* and human mutant APP, but also the resulting behavioral deficits.

Previous research from our lab has characterized the neuropathological and behavioral profiles of various transgenic mouse models. In one study, Hartman et al. (2001) crossed *APOE* KO mice with transgenic mice expressing either human E3 or human E4 to examine the effects of human *APOE* in the absence of endogenous murine *APOE*. Compared to human *APOE*, murine *APOE* is most similar to E3 and 4, as shown in Holtzman (2004) where E3 and 4 mice developed diffuse and neuritic plaques similar to those seen in the presence of murine *APOE*.

PDAPP$^{+/-}$ mice in the presence or absence of murine *APOE* all began to develop Aβ deposits by 9 months of age (D. Holtzman, 2004). Another study using an APP transgenic mouse model of AD showed that the absence of murine *APOE* does not result in a delay in the onset of Aβ deposition (David M Holtzman et al., 1999), but it does result in a decrease in the level of Aβ deposition (K. R. Bales et al., 1997).

Hartman et al. (2001) was the first to report the behavioral phenotyping of GFAP-E3 and GFAP-E4 transgenic mice in which both isoforms of human *APOE* are expressed in glia. Glia is the predominant cell type in the CNS that synthesizes *APOE* in mammals. The *GFAP* (glial fibrillary acidic protein) gene provides information for the GFAP protein. This protein is an intermediate filament (cytoskeletal components, responsible for cell shape, division, and function) that is expressed by numerous cell types of the CNS including astrocytes (Jacque et al., 1978; Venkatesh et al., 2013).

Hartman et al. (2001) carried out a longitudinal study where E3 and E4 mice were compared with *APOE* KO and WT mice (all male). Various behavioral measures were

conducted. ApoE4 mice appeared to be the most "emotionally reactive" compared to E3, *APOE* KO, and WT mice. ApoE4 mice were also significantly impaired on working memory tasks. The presence of behavioral impairments and the lack of AD-like neuropathology displayed in the E4 mice suggests that the *APOE* genotype plays a role in the AD-related cognitive impairments in humans, which may be distinct from any effects on Aβ structure and deposition.

Hartman et al. (2002) then looked at how *APOE* and TBI affect the development of AD. The model consisted of PDAPP mice expressing human E3*,* E4, or no *APOE* subjected to unilateral cortical impact injury. The TBI was induced at 9-10 months of age and monitored for 3 months. It was previously found that PDAPP mice expressing human ε*3* and ε4 do not develop Aβ deposition until about 15 months of age (D M Holtzman et al., 2000). Hartman et al. (2002) found that, following TBI, a high percentage of brain-injured PDAPP:ε4 mice had Aβ deposition by 12-13 months of age. The TBI appeared to accelerate Aβ deposition in the form of amyloid in the presence of human ε4 to a greater extent than ε3 (Richard E Hartman et al., 2002). Also, they found that the PDAPP mice that did not express human *APOE* had significantly greater Aβ load than those that expressed ε3 and ε4. However, the Aβ deposits were non-fibrillar, consisting of only thioflavin-S-negative Aβ (Richard E Hartman et al., 2002).

Thioflavin-S-positive Aβ was only present in the dentate gyrus in the PDAPP:ε4 mice, specifically 44% of the 56% of the Aβ-immunoreactive deposits (Richard E Hartman et al., 2002). Thioflavin-S-positive Aβ suggests the presence of fibrillar amyloid deposition. The dentate gyrus is located in the inner portion of the hippocampus, as depicted by the letters "DG" in Figure 4. No PDAPP:ε3 mice had fibrillar amyloid

deposition. There also existed no significant difference in the amount of brain tissue or cell loss across the groups. Given that the presence or absence of *APOE* isoforms was the only difference between the groups of PDAPP mice, they found that the isoform-specific *APOE*-Aβ interactions contribute to the premature development of AD pathology (Richard E Hartman et al., 2002).

In heterozygous PDAPP mice, Aβ deposition in the molecular layer of the dentate gyrus was compared in mice expressing no *APOE*, murine *APOE*, or various isoforms of human *APOE* (Fagan et al., 2002). Aβ deposition was the most robust in the molecular layer of the dentate gyrus in animals expressing mouse *APOE* and increased with age (Figure 5). PDAPP mice expressing E4 also displayed increases in Aβ deposition, but to a much less magnitude than that observed in the murine *APOE* mice. The amount of Aβ deposition in the dentate gyrus was low in the absence of *APOE* up to 18 months, but increased by 21 months (Fagan et al., 2002).

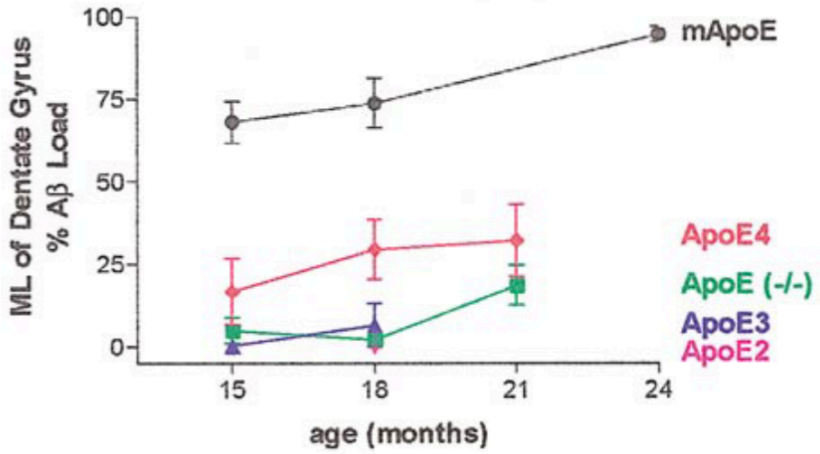

Figure 5. The Aβ deposition in the molecular layer of the dentate gyrus, in heterozygous PDAPP mice, was compared in mice expressing no apoE, murine apoE, or various isoforms of human apoE. PDAPP mice expressing apoE4 also displayed increases in Aβ deposition, but to a much less magnitude than that observed in the murine apoE mice (Fagan et al., 2002).

Hartman et al. (2002) differs from the current study in that a TBI is induced and that the transgenic mice were not "targeted" replacement/KI mice. Also, they found no significant difference in the amount of cell loss across groups (PDAPP:ε3, PDAPP:ε4, PDAPP:ε-/-). This lack of difference may be due to the fact that the TBI may have had a larger affect on the PDAPP:ε3 and PDAPP:ε-/- mice, diminishing the difference of cell loss across groups. TBI was not induced in the current study, allowing us to investigate the cellular loss solely based on the double transgenic mice, with no other interfering variables.

Hartman et al. (2005) then characterized Aβ plaque load and learning deficits in the PDAPP transgenic mouse model. They found that the PDAPP mice had a substantial age-dependent decline in learning ability. To determine whether the age-related spatial learning deficits were due to the Aβ accumulation, Hartman et al. (2005) treated WT and PDAPP mice with a passive anti-Aβ antibody (10D5), which specifically targets Aβ by

binding to amino acids 3-6 in the n-terminus (towards the beginning of the Aβ peptide) (Hyman, Tanzi, Marzloff, Barbour, & Schenk, 1992). Over several weeks of treatment with 10D5, aged PDAPP mice improved in hippocampal long-term potentiation and behavioral performance with substantial Aβ burden. This suggests that Aβ had in fact contributed to the age-dependent learning deficits displayed in PDAP mice. Should our first hypothesis (PDAPP:E4 mice will show more Alzheimer's-like pathology in the hippocampus and dorsal cortex than the PDAPP:E3 mice) be correct, the findings from Hartman et al. (2005) would aid in supporting the third hypothesis (Alzheimer's-like pathology will be positively correlated with the behavioral deficits displayed in the mice).

The advantage to using this mouse model over the aforementioned models is establishing a baseline behavioral and neuropathological data for a new double-transgenic mouse model for AD. This study has important implications for understanding the effects of new therapeutic medications on the behavior of those suffering from AD. Various therapeutic techniques have already shown productive in AD mouse models. When administered early in the AD course of a Tg2576 transgenic mouse model, ibuprofen, a non-steroidal anti-inflammatory drug (NSAID), has led to a significant delay in amyloid deposition (G. P. Lim et al., 2000). It was also found that antioxidants including melatonin (Matsubara et al., 2003), curcumin (G. P. Lim et al., 2001), vitamin C/E (Harkany et al., 1999), blueberry extract (Joseph et al., 2003), and pomegranate juice (Richard E Hartman et al., 2006) reduced Aβ levels in Tg2576 mice.

Lastly, *APOE* mimetics have shown to be effective in AD mouse models. ApoE mimetics are small peptides, derived from the receptor-binding region of *APOE*, that mimic the neuroprotective, anti-oxidant, and anti-inflammatory properties of intact *APOE*

(Aono et al., 2003; Croy, Brandon, & Komives, 2004; D T Laskowitz et al., 2001; Lynch et al., 2003, 2005; McAdoo et al., 2005). ApoE133-149 (COG133) is a 17-amino-acid peptide that has demonstrated *in vivo* anti-inflammatory and neuroprotective activity in animal models of AD (Vitek et al., 2012), TBI (Lynch et al., 2005), and hypoxic-ischemic injury (HIE; McAdoo et al., 2005). Starting at 9 months, Vitek et al. (2012) administered COG133 subcutaneously 3 times per week for 3 months to CVND-AD (SwDI-APP/NOS2$^{-/-}$) transgenic mice. Following treatment, these animals maintained significantly more of their neurons, grew fewer plaques and neurofibrillary tangles, and navigated better in the radial arm water maze than did untreated animals (Vitek et al., 2012). COG1410, a shorter analogue to COG133, is a 12-amino-acid peptide that is also active in animal models, but with increased potency and extended latency between injury and time to treatment (D. T. Laskowitz, Fillit, Yeung, Toku, & Vitek, 2006; Daniel T Laskowitz et al., 2007). ApoE mimetics represent a novel therapeutic strategy to neurological disorders including AD.

CHAPTER FOUR

DESIGN

Animals

Transgenic mice expressing human amyloid precursor protein (APP) with a mutation (V717F) that causes early onset familial Alzheimer's disease (AD) were crossbred with transgenic mice expressing human apolipoprotein E (E3 or E4) to create double-transgenic mice (PDAPP:E3 and PDAPP:E4). Initially, 30 PDAPP:E3 and 16 PDAPP:E4 double-transgenic mice, 21-24 months of age, were obtained from Eli Lilly Corporation. They were housed on a 12:12 light:dark schedule and fed standard rodent chow and water *ad libitum*. These mice underwent a battery of behavioral and cognitive tests that was approved by Loma Linda University Institutional Animal Care and Use Committee (IACUC), including the rotarod (assessing balance and sensorimotor coordination), open field (assessing general activity levels and movement patterns), elevated zero maze (assessing general anxiety levels), and water maze (assessing learning and memory). The brains of a subset of these mice were assessed in the current study (n = 5 PDAPP:E3; n = 4 PDAPP:E4; all males).

Tissue Processing and Histology

Animals were sacrificed via intra-cardiac perfusion. The brains were removed and immersed in paraformaldehyde, fixed in a 30% sucrose solution at 4ºC for about 18 months, and then frozen on dry ice and stored at -20ºC. Coronal sections, 50 microns thick, were sectioned from the brains' anterior (prefrontal cortex [PFC]) to the posterior (cerebellum) using a cryostat. Coronal sections are taken along the frontal plane of the

brain, dividing the brain into anterior and posterior portions. The free-floating sections were preserved by placing them in a solution of 400 mL phosphate buffer solution (PBS), 400 mL sodium azide (NaN_3), and 3,000 mL double distilled water (ddH_2O). Each well contained four to five anterior, middle, and posterior slices from each brain.

A subset of free-floating sections was stained for diffuse early stage Aβ deposits (pre-clinical) using HJ3.4, an antibody that binds to the 1st 13 amino acids of Aβ's N-terminus (i.e., Aβ1-13) and has a fluorescent tag (Tran, Sanchez, Esparza, & Brody, 2011). These plaques lack a morphologically identifiable substructure, resembling a ball of cotton (Dickson & Vickers, 2001). HJ3.4 staining began with thoroughly washing the sections in PBS. To enhance antigen (e.g., Aβ) binding, the sections were blocked at room temperature in a humid chamber in 1% bovine serum albumin (BSA) in PBS 2 x 10 minutes for 90 minutes. BSA enhances antigen detection by binding to and blocking other epitopes (binding sites). The sections were then incubated overnight at 4ºC with HJ3.4 antibody (1:1000) in 0.25% BSA/0.25% TritonX-100 in PBS. TritonX-100 is a detergent used to enhance antibody penetration during immunohistochemistry by acting as a surfactant (reduces surface tension of a liquid in which it is dissolved). Following another thorough wash in PBS 2 x 10 minutes and ddH_20 2 x 1 minute (to remove PBS salts), sections were mounted on Superfrost slides and allowed to dry. About 20 microliters of Vectashield, a mounting medium that prevents photobleaching, was placed on each section and disbursed equally with a cover slip placed over it. Once dry, clear nail polish was used around the edges of the coverslip to hold it in place. The Vectashield contained 4',6-diamidino-2-phenylindole (DAPI), a fluorescent stain that stains DNA in cell nuclei. DAPI was used to analyze the cellular count of the tissue. The slides were

then stored in 4ºC. Figure 6 illustrates the HJ3.4 staining of diffuse Aβ in the hippocampus of a PDAPP:E4 mouse from the current study.

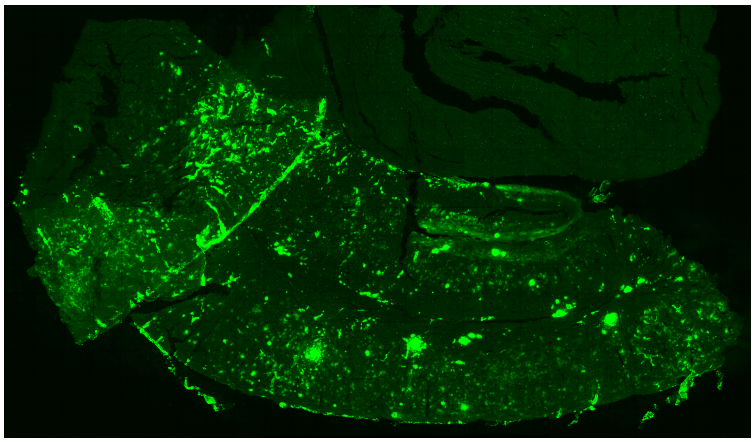

Figure 6. HJ3.4 staining of diffuse deposits in the hippocampus of a PDAPP:E4 double transgenic mouse from the current study.

Another subset of brain sections was stained for fibrillar (late stage "amyloid") Aβ plaques using thioflavin-S. These mature amyloid plaques have a dense core (A. E. Roher et al., 2004) that can be identified by their compacted central mass of β-amyloid (Dickson & Vickers, 2001) surrounded by neuritic processes (i.e., injured axons/dendrites). Figure 7 illustrates fibrillar Aβ deposits stained with thioflavin-S in transgenic mice. Figure 8 illustrates fibrillar Aβ as well, in pomegranate-treated mice.

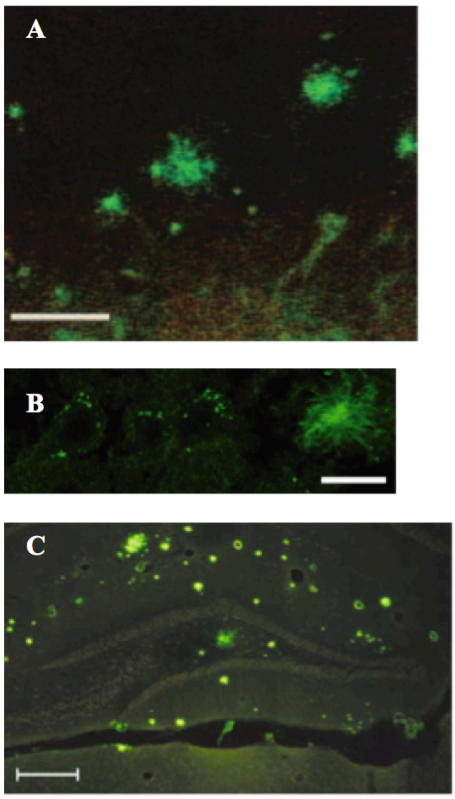

Figure 7. A. Thioflavin-S staining compact deposits in CA1 of the hippocampus of $APP^{V717F+/+}$ *APOE*$^{-/-}$ transgenic mice. Scale bar = 100µm (Irizarry et al., 2000). B. Thioflavin-S positive amyloid plaque. Scale bar = 20 µm (Oakley et al., 2006). C. Coronal sections of the dorsal hippocampus stained with Thio-S. (Groen et al., 2003).

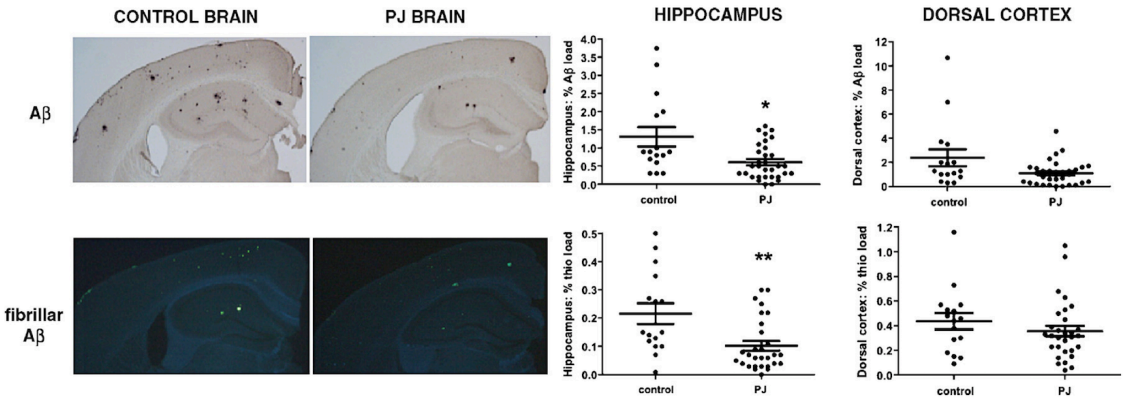

Figure 8. Brain Aβ load. Brains from mice treated with control and pomegranate juice. Mice treated with pomegranate juice had significantly total Aβ (top row) and fibrillar (thioflavin-S-positive) Aβ (bottom row) in the hippocampus and dorsal cortex than the control mice (Richard E Hartman et al., 2006).

Assessing these two stages of plaques not only allows us to differentiate between early and end stages of Aβ plaques in AD, but to determine whether our hypotheses hold true for both stages of Aβ plaques.

Thioflavin-S staining began by thoroughly washing sections in PBS and then in ddH_2O to remove PBS and acclimate to the ddH_2O. Ethanol (EtOH) was used to dehydrate the sections. The sections were then stained with a solution of 1% thioflavin-S. To clean any excess stain, the sections were then washed with ddH_2O. After staining for thioflavin-S, the sections were mounted onto slides and allowed to dry. About 20 microliters of Vectashield was placed on each section and disbursed equally with a cover slip placed over it. Once dry, clear nail polish was used around the edges of the coverslip to hold it in place. The slides were then stored in 4ºC.

Aβ Quantification and Cellular Density

For quantification of Aβ, histology slides were initially evaluated using a Zeiss LSM 710 confocal microscope, provided by the Loma Linda University School of Medicine Advanced Imaging and Microscopy Facility. Due to unforeseen technical difficulties, imaging of Aβ deposits was completed using an Olympus FluoView FV-1000 confocal microscope, provided by Dr. Hongwei Dong of Zilkha Neurogenetic Institute at the Keck School of Medicine of University of Southern California.

Images of the entire extent of the hippocampus and dorsal cortex (defined as cortical tissue directly over the dorsal hippocampus) were assessed at 40x magnification. ImageJ, an image processing and analysis software, and Adobe Photoshop were used to quantify fibrillar and total Aβ load, defined as the percentage of each tissue section

covered by deposits. The Aβ load was calculated by dividing the total area of tissue covered by deposits (quantified in ImageJ) by the total area of the region of interest (hippocampus or dorsal cortex; quantified in Adobe Photoshop).

For quantification of cellular density, histology slides were evaluated using a Keyence BZ-X700 Fluorescence Microscope, provided by Dr. Andre Obenaus' Non-Invasive Imaging Laboratory in the Radiation Biology Program at Loma Linda University. Images of the hippocampus and dorsal cortex were assessed at 40x magnification. Images were taken of three areas in the hippocampal CA1 region (CA1-A, CA1-B, and CA1-C; Figure 9a) at three depths (approximately every 7.5 μm below the surface of the 50 μm thick tissue sections; Figure 10). Cells were quantified for each layer using the "cell counter" plugin in ImageJ and summed together. This sum was then divided by the volume of the CA1 to determine the cellular density in cells/mm^3. The same procedure was done for the dorsal cortex region (DCx-A, DCx-B, and DCx-C; Figure 11a).

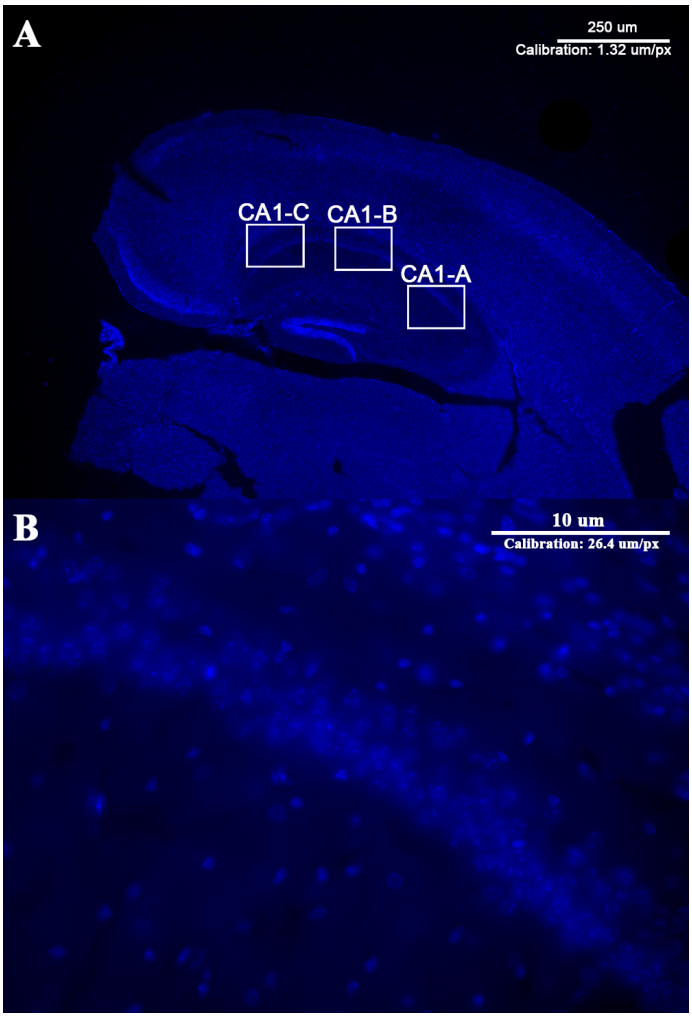

Figure 9. A. CA1-A, CA1-B, and CA1-C along the CA1 region of animal "5-4," a PDAPP:E4 mouse. Image taken at 2x magnification. ***B.*** CA1-B of the hippocampus of animal "5-4." Image taken at 40x magnification. Blue dots indicate stained cell nuclei.

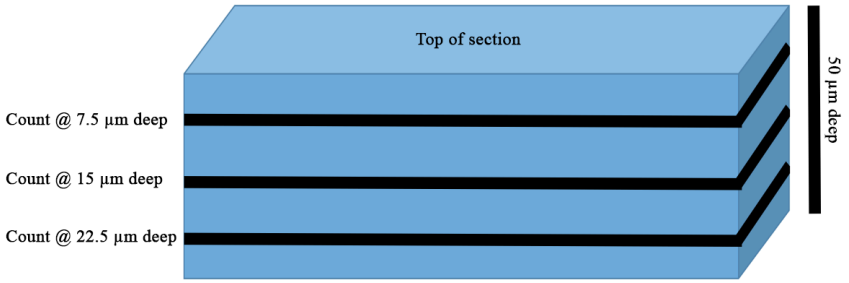

Figure 10. This is an image of a cross-section of a brain slice to help visualize how the brain sections were analyzed in the current study. In the current study, the brain section was 50 um thick. Images were analyzed at three depths (approximately every 7.5 µm below the surface of the section).

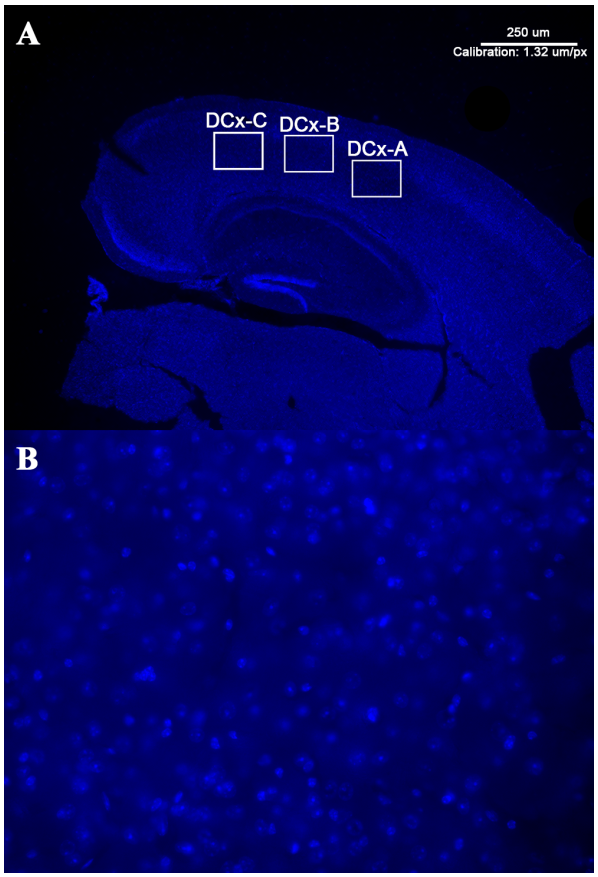

Figure 11. A. DCx-A, DCx-B, and DCx-C in the dorsal cortex (DCx) of animal "5-4," a PDAPP:E4 mouse. Image taken at 2x magnification. ***B.*** DCx-B of animal "5-4." Image taken at 40x magnification. Blue dots indicate stained cell nuclei.

Statistical Analysis

IBM SPSS Statistics version 20 will be used to analyze the collected data. All data will be presented as mean +/- standard error of the mean (SEM), and graphs will be generated using GraphPad version 6.0d. An α-level of .05 will be used for all significance tests. For the Aβ load analysis, independent-samples t-tests were conducted to compare the total Aβ load in HJ3.4-stained PDAPP:E4 and PDAPP:E3 brain sections in the hippocampus and dorsal cortex. Independent-samples t-tests were also conducted to compare fibrillar Aβ (amyloid) load in thioflavin-S-stained brain sections. Although two *t*-tests were performed, a bonferroni correction was not used since previous research has shown that two *t*-tests are not enough to cause a real problem with type I error.

An independent samples *t*-tests was also used to test between-group differences of cellular density of PDAPP:E4 and PDAPP:E3 mice in the CA1 and dorsal cortex. An independent t-test was first conducted to analyze the total cellular density from two areas of the CA1 or dorsal cortex as a function of *APOE* type. A multivariate analysis of variance (MANOVA) was then conducted to determine whether cell densities between PDAPP:E4 and PDAPP:E3 mice in the CA1 or dorsal cortex still differed when the two areas within the region were analyzed separately. For the CA1 analyses, an independent samples t-test was then conducted to determine whether there was a significant difference between the cellular density in PDAPP:E4 and PDAPP:E3 mice in only CA1-C.

Lastly, for determining whether there is a correlation between neuropathology and behavioral deficits, bi-correlation analysis will be used to measure the strength and direction of the relationship between neuropathology and behavioral deficits.

A total of 9 animals (E3 $n = 5$; E4 $n = 4$) will be assessed. Sections stained with HJ3.4 and thioflavin-S will be included for each animal. Independent-samples t-tests were conducted to compare the total Aβ load in HJ3.4-stained PDAPP:E4 and PDAPP:E3 brain sections in the hippocampus and dorsal cortex. The probability that the independent samples *t*-test will find statistically significant differences in Aβ load and cellular count in the two groups, given that a difference exists, is 71% (*power* = 0.71; *effect size* = 0.8; $\alpha = 0.05$; $n = 9$). Given that a specific direction is hypothesized (i.e., that PDAPP:E4 mice will show significantly more Aβ staining than PDAPP:E3 mice) a one-tailed test will be used. Regarding the third hypothesis, power analysis showed that studying nine animals would give a statistical power sufficient to have a 97% chance of detecting a correlation between neuropathology and behavioral deficits. Outliers, defined as any data point that fell within a distance of three times the interquartile range from the first and third quartiles, were removed.

CHAPTER FIVE

RESULTS

Given that the following analyses were based on planned comparisons, Bonferroni corrections were not used for Hypothesis One, Two, or Three.

Hypothesis One

One PDAPP:E3 mouse was removed from the overall Aβ analysis for being 1.6 standard deviations above the hippocampal mean and 1.8 standard deviations above the dorsal cortex mean. Two PDAPP:E3 mice were removed from the dorsal cortex fibrillar Aβ analysis, one for being 1.6 standard deviations above the mean and another one for being 1.1 standard deviations below the mean.

The hypothesis that PDAPP:E4 mice would have more Aβ plaques (total and fibrillar) in their brains than PDAPP:E3 mice was partially confirmed. A trend was observed in the hippocampus suggesting that PDAPP:E4 mice had more total Aβ load (defined as the % area stained by HJ3.4) than PDAPP:E3 mice (PDAPP:E4 M=3.023%, SD=2.232; PDAPP:E3 M=.875%, SD=.738; t(6)=-1.827, p>.1; Figure 12a). A significant difference was found in the dorsal cortex suggesting PDAPP:E4 mice had more total Aβ load than PDAPP:E3 mice (PDAPP:E4 M=2.19%, SD=1.055; PDAPP:E3 M=.533%, SD=.230; t(6)=-3.069, p<.02; Figure 12b**)**. However, levels of fibrillar Aβ (amyloid) did not differ between the groups.

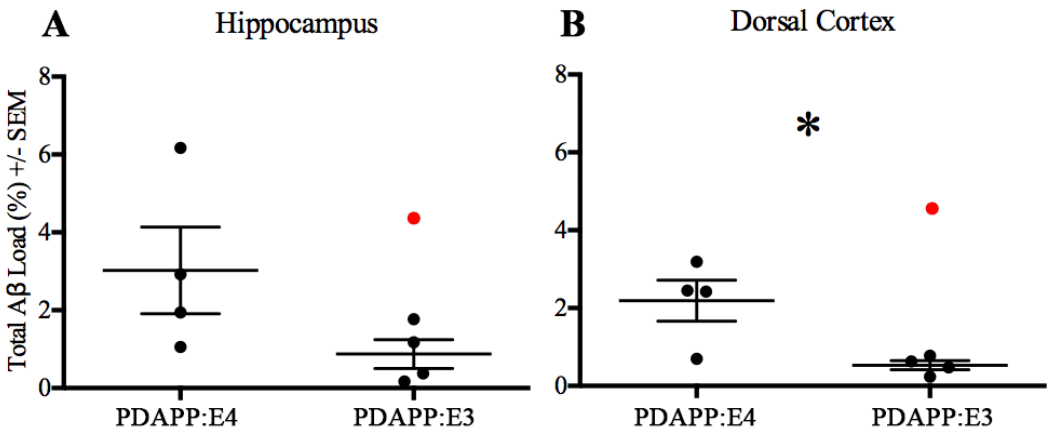

Figure 12. A. There was no significant difference in total Aβ hippocampal load between HJ3.4-stained PDAPP brain sections expressing human E4 and those expressing human E3. ***B.*** HJ3.4-stained PDAPP:E4 brain sections had a significantly greater total Aβ load in the dorsal cortex than PDAPP:E3 sections. Note: *p<.025. Data points in red are outliers excluded from analysis.

Interestingly, amyloid load in both the hippocampus and dorsal cortex was lower in PDAPP:E4 mice than in PDAPP:E3 mice, although the difference was not significant (Hippocampus: PDAPP:E4 *M*=.5%, *SD*=0.215; PDAPP:E3 *M*=1.42%, *SD*=.991; t(7)=1.8, *p*>.10; Dorsal cortex: PDAPP:E4 mice *M*=.833%, *SD*=0.353; PDAPP:E3 *M*=.897%, *SD*=.055; t(5)=.305, p>.6; Figures 13a and 13b, respectively).

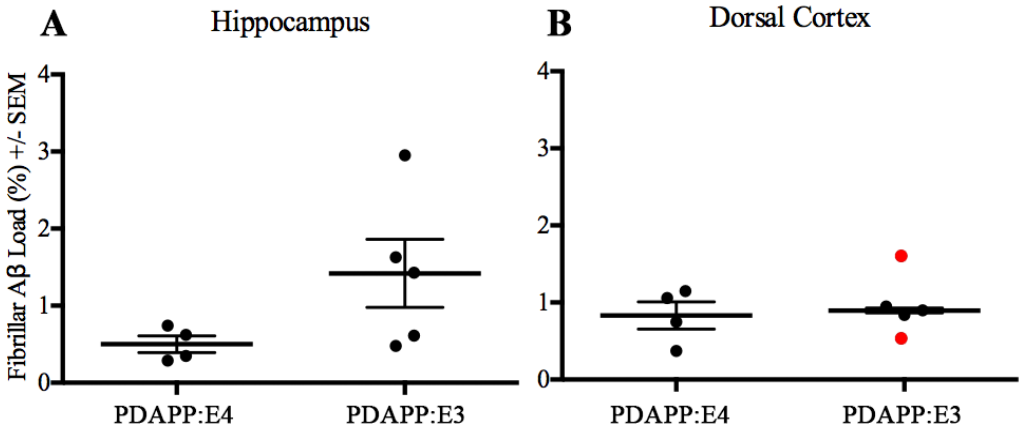

Figure 13. A. No significant difference in fibrillar Aβ load in the hippocampus or ***B.*** dorsal cortex only out of thioflavin-S-stained animals between PDAPP:E4 and PDAPP:E3 brain sections. Data points in red are outliers excluded from analysis.

Given that only one statistically significant difference was found in assessing whether expression of human *APOE* will alter plaque load in the hippocampus and dorsal cortex of PDAPP mice, the fibrillar Aβ:total Aβ ratio was analyzed. The previously excluded outliers were included in this analysis to assess the overall fibrillar β:total Aβ ratio for each group of mice. The ratios were analyzed by calculating the sum of the fibrillar Aβ load and total Aβ load in the hippocampus and dorsal cortex to determine whether there was a statistically significant difference between PDAPP:E4 and PDAPP:E3 mice (Table 3).

Table 3. Overall fibrillar Aβ:total Aβ ratio.

Genotype	Hippocampus	Dorsal Cortex	Both Regions
PDAPP:E4	0.17	0.38	0.26
PDAPP:E3	0.93	0.74	0.84

Findings indicated both groups of mice had a higher total Aβ load than fibrillar Aβ load, as expected, in both the hippocampus and dorsal cortex. Also, in both regions, a trend

indicates that PDAPP:E4 mice have a lower fibrillar Aβ:total Aβ ratio (Ratio=0.26) than PDAPP:E3 mice (Ratio = 0.84).

To summarize the results of Hypothesis One, PDAPP:E4 mice had more total Aβ than PDAPP:E3 mice in both the hippocampus and dorsal cortex, although only the cortical difference was statistically significant. In contrast, PDAPP:E4 mice had less fibrillar Aβ than PDAPP:E3 mice in both the hippocampus and dorsal cortex, although neither difference was statistically significant. Lastly, a trend indicates that PDAPP:E4 mice have a lower fibrillar Aβ:total Aβ than PDAPP:E3 mice.

Hypothesis Two

The hypothesis that PDAPP mice that also express human E4 will have a lower cellular density in their CA1 and dorsal cortices than PDAPP mice that also express human E3 was partially confirmed. Specifically, PDAPP:E4 mice had a lower cellular density than PDAPP:E3 mice in the CA1 and dorsal cortex, but the difference was not statistically significant.

Since the cellular densities for CA1-A (the most lateral area in the CA1; Figure 9a) were very similar between PDAPP:E4 and PDAPP:E3 mice (not statistically different), only CA1-B and -C were included in the analyses. The cellular density from CA1-B and -C were summed together and then divided by the CA1 volume to get the total CA1 cellular density. As hypothesized, PDAPP mice that also expressed E4 had a lower cellular density in the CA1 than PDAPP mice that also expressed E3 (PDAPP:E4 M=201,132.61 cells/mm^3, SD=49,021.35; PDAPP:E3 M=259,563.30 cells/mm^3, SD=51,498.67; t(6)=1.56, p<.17), although the difference was not statistically significant.

When determining whether the cellular density between PDAPP:E4 and PDAPP:E3 mice in the CA1 still differed when CA1-B and -C were analyzed separately, PDAPP:E4 mice still had a lower cellular density in CA1-B and -C separately than PDAPP:E3 mice (CA1-B: PDAPP:E4 M=108,034.20 cells/mm^3, SD=16,741.02; PDAPP:E3 M=126,861.45 cells/mm^3, SD=12,967.54; CA1-C: PDAPP:E4 M=93,098.41 cells/mm^3, SD=14,774.98; PDAPP:E3 M=132,701.85 cells/mm^3, SD=11,444.65; $F(2,5)=2.23$, $p<.21$; Wilk's $\Delta=.53$; Figure 14). However, the difference was not statistically significant.

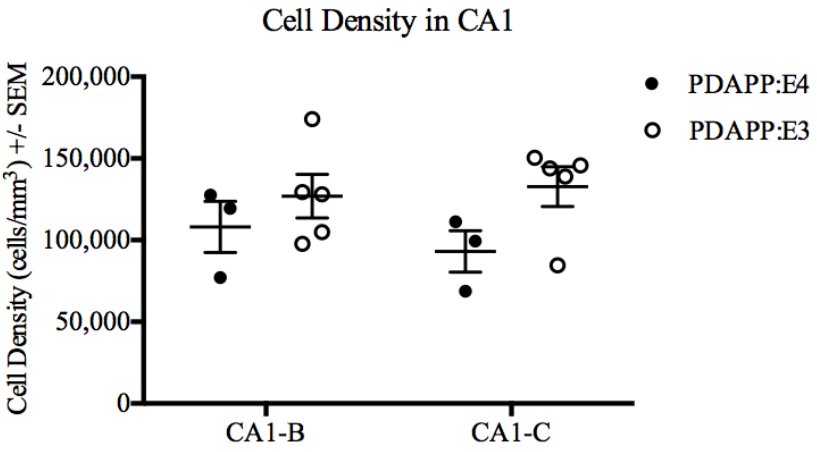

Figure 14. PDAPP:E4 mice had a lower cellular density in CA1-B and -C of the hippocampus separately than PDAPP:E3 mice, but the difference was not statistically significant.

When assessing the difference between the cellular density in PDAPP:E4 and PDAPP:E3 mice in CA1-C only, findings suggested that PDAPP:E4 mice still had a lower cellular density in CA1-C (medial), but the difference was not statistically

significant (PDAPP:E4 M=93,098.41 cells/mm^3, SD=21,951.43; PDAPP:E3 M=132,701.85 cells/mm^3, SD=27,228.98; t(6)=2.12, p<.08; Figure 15).

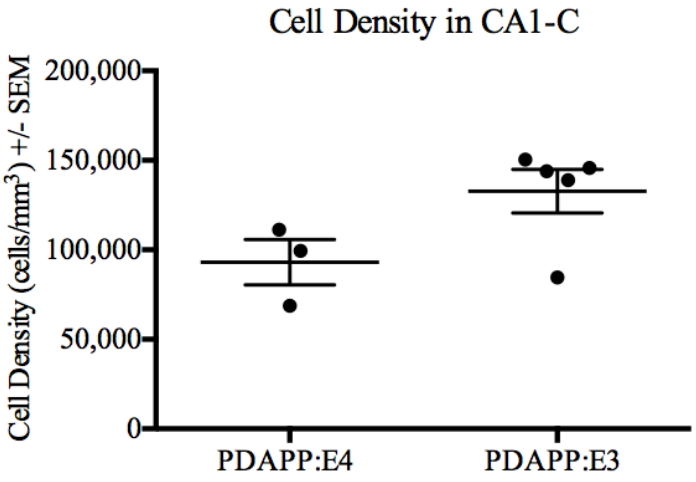

Figure 15. PDAPP:E4 mice had a lower cellular density than PDAPP:E3 mice in CA1-C of the hippocampus, but the difference was not statistically significant.

A non-parametric test (Kruskal Wallis) was conducted to determine whether a statistically significant difference could be found with CA1-B and -C combined when accounting for the small sample size. The Kruskal Wallis test corroborated these results (CA1-B: $\chi^2(1)=1.09$, p<.30; CA1-C: $\chi^2(1)=2.69$, p<.11).

The cellular density in the dorsal cortex was assessed next. Since cellular densities for DCx-C (the most medial area in the dorsal cortex; Figure 11a) were very similar between PDAPP:E4 and PDAPP:E3 mice (not statistically different), only DCx-A and -B were included in the analyses. The cellular density from DCx-A and -B were summed and then divided by the total cortical volume to get a total cortical cellular density. PDAPP mice that also expressed E4 had a lower cellular density in the dorsal

cortex than PDAPP mice that also expressed E3 (PDAPP:E4 *M*=461,209.30 cells/mm^3, *SD*=146,835.99; PDAPP:E3 *M*=533089.43 cells/mm^3, *SD*=218,760.67; t(6)=.50, *p*<.*64*), although the difference was not statistically significant.

Next, we determined whether cellular density between PDAPP:E4 and PDAPP:E3 mice in the dorsal cortex differed when DCx-A and -B were analyzed separately. Transformational analyses were used to include the previously excluded outliers by taking the square root of each value. Findings suggested a trend such that PDAPP:E4 mice had a lower cellular density than PDAPP:E3 mice in DCx-A and DCx-B (DCx-A: PDAPP:E4 *M*=459.32 cells/mm^3, *SD*=47.18; PDAPP:E3 *M*=503.51 cells/mm^3, *SD*=36.54; DCx-B: PDAPP:E4 *M*=491.61 cells/mm^3, *SD*=72.28; PDAPP:E3 *M*=507.82 cells/mm^3, *SD*=55.99; F(2,5)=.33, *p*>.73; Wilk's Δ=.883; Figure 16), although the difference was not statistically significant.

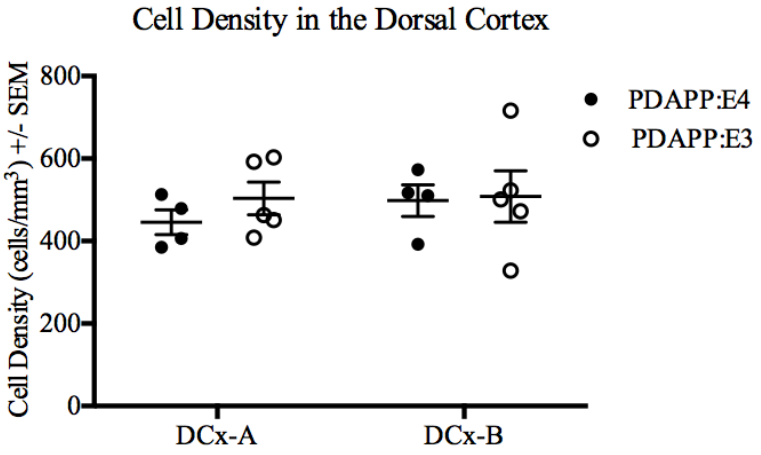

Figure 16. There were no statistically significant differences in cellular density between PDAPP:E4 and PDAPP:E3 mice within DCx-A and –B following a transformational analysis. However, a trend suggested that PDAPP:E4 mice had a lower cellular density than PDAPP:E3 mice.

A non-parametric test (Kruskal Wallis) was conducted to determine whether a statistically significant difference could be found when accounting for the small sample size. These results also suggested no statistically significant difference in the cellular density in DCx-A or -B between PDAPP:E4 and PDAPP:E3 mice (DCx-A: $\chi^2(1)=.20$, $p>.65$; DCx-B: $\chi^2(1)=.02$, $p>.88$).

To summarize the results of Hypothesis Two, PDAPP:E4 mice had a lower cellular density than PDAPP:E3 mice in the CA1 and dorsal cortex, although not statistically significant (Table 4).

Table 4. Means and standard deviations from Hypothesis Two analyses.

		PDAPP x ApoE4 Cell Count		PDAPP x ApoE3 Cell Count		
Tissue Region	Area of Region	*M*	*SD*	*M*	*SD*	*p-value*
Hippocampus	CA1-B and -C combined	430	101.43	558.6	58.76	0.06
	CA1-B only	237.33	61.81	279.6	21.45	0.20
	CA1-C only	192.67	40.5	279	54.52	0.06
Dorsal Cortex	DCx-A and -B combined	1101.33	45.08	961.8	426.36	0.51
	DCx-A only	547	44.17	573.2	125.29	0.69
	DCx-B only	587.67	46.09	538.2	195.45	0.69

Note. ¥ = Transformational analyses were conducted such that the square root was taken of each value

Hypothesis Three

The hypothesis that mice with more Alzheimer's-like neuropathology (specifically Aβ plaques and/or lower cellular density) will have worse behavioral deficits was confirmed.

Correlation analyses were conducted to determine whether the degree of neuropathology was correlated with behavioral performance. In PDAPP:E3 mice, a higher amyloid load in the dorsal cortex was associated with less activity in the open field tasks (average distance traveled: r=.95, p<.02; Figure 17; average duration traveled: r=.92, p<.03; Figure 18).

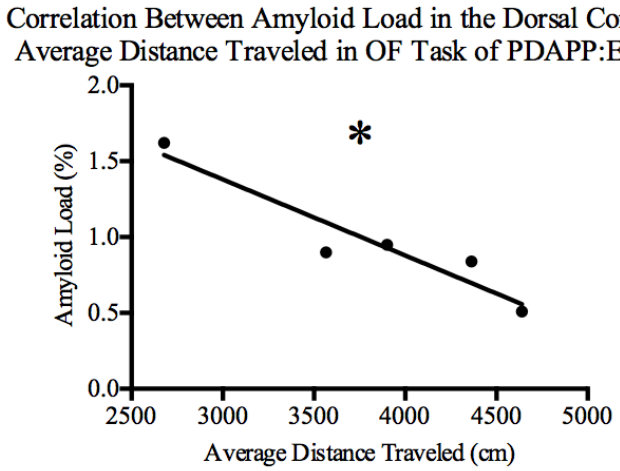

Figure 17. In PDAPP:E3 mice, a higher amyloid load in the dorsal cortex was associated with less distance traveled in the open field task (r=.95, p<.02).

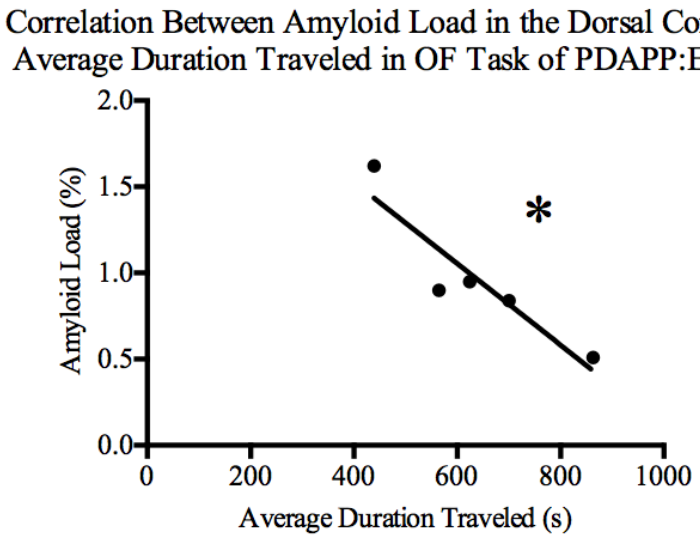

Figure 18. In PDAPP:E3 mice, a higher amyloid load in the dorsal cortex was associated with time spent moving in the open field task (r=.92, p<.03).

Interestingly, across both groups of mice, a higher amyloid load in the dorsal cortex trended toward an association with better performance on the rotarod task, although the correlation was not statistically significant (r=.65; p>.05; Figure 19a). When breaking up the regions by stain and *APOE* allele, other statistically significant correlations were found. Specifically, a higher total Aβ load in the hippocampus among PDAPP:E3 mice was associated with better performance on the rotarod when rotating at a steady rate (r=.88, p<.05; Figure 19b). Among PDAPP:E4 mice, a higher total Aβ load in the dorsal cortex was associated with worse performance on the rotarod when rotating at an accelerated rate (r=-.97, p<.04; Figure 19c) and when rotating at a faster accelerated rate (r=-.97, p<.03; Figure 19d), following transformational analysis consisting of taking the square root of each value.

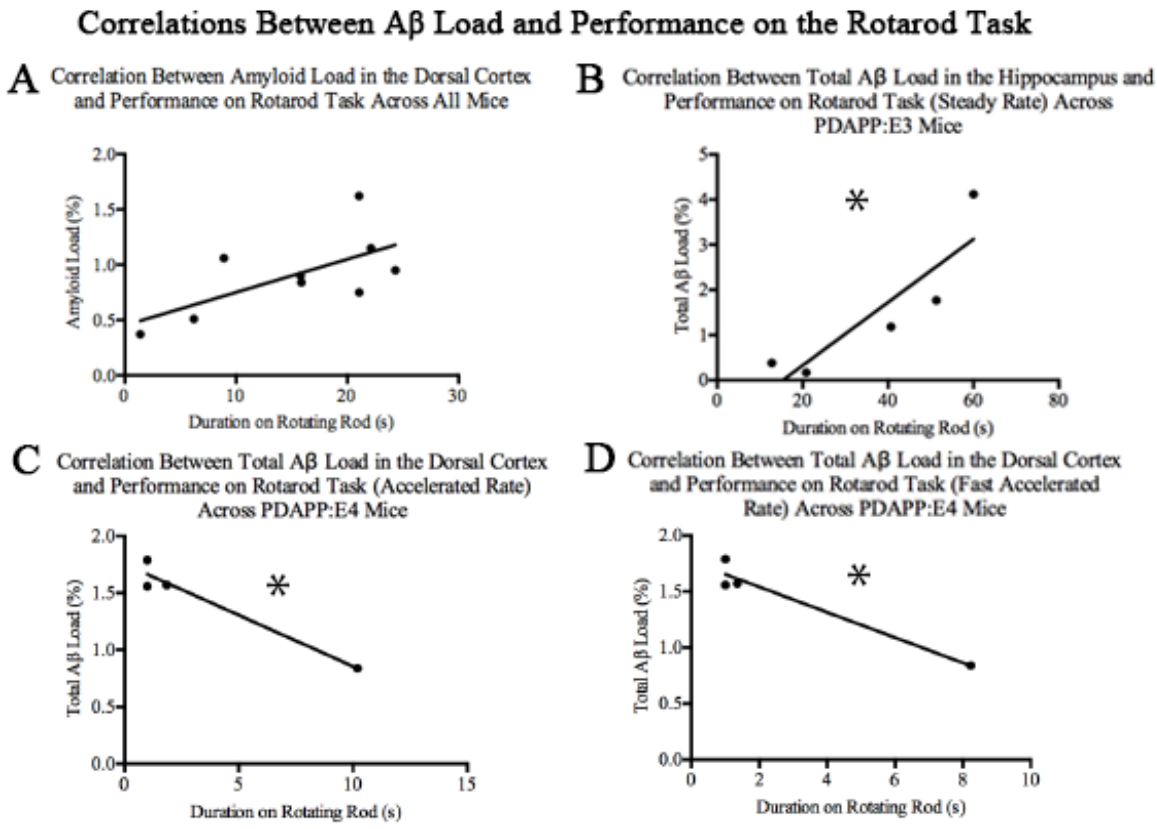

Figure 19. A. Across both groups of mice, a higher amyloid load in the dorsal cortex trended toward an association with better performance on the rotarod task, although the correlation was not statistically significant (r=.65; p>.05). ***B.*** Among PDAPP:E3 mice, a higher total Aβ load in the hippocampus was statistically significantly correlated with better performance on the rotarod when rotating at a steady rate (r=.88, p<.05). ***C.*** Among PDAPP:E4 mice, a higher total Aβ load in the dorsal cortex was statistically significantly correlated with worse performance on the rotarod when rotating at an accelerated rate (r=-.97, p<.04) and ***D.*** when rotating at a faster accelerated rate (r=-.97, p<.03) following transformational analyses consisted of taking the square root of each value.

There were no significant correlations between total Aβ load or amyloid load in the dorsal cortex or hippocampus with water maze or zero maze performance.

Correlation analyses were then conducted to determine whether cellular densities in the CA1 or dorsal cortex were correlated with behavioral deficits. Findings suggested a statistically significant correlation, such that a lower cellular density in the CA1 was

associated with worse performance during the probe trials (a lower percent time in the target quadrant; r=-.81, p<.02; Figure 20) of the water maze. Additionally, a trend suggested that lower cellular density in the CA1 was associated with worse performance (traveling a greater distance) in the cued task (r=-.69, p<.059; Figure 21) and in the spatial task (r=-.63, p<.094; Figure 22). There were no significant correlations between the cellular density in the CA1 and any other behavioral performance. There were no significant correlations between total cellular density in the dorsal cortex and any behavioral performance.

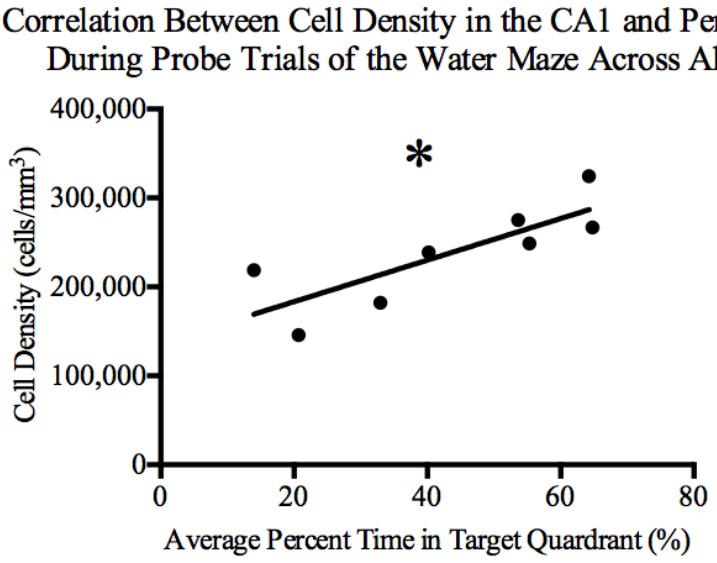

Figure 20. Across both groups of mice, lower cellular density in the CA1 was associated with worse performance (lower percent time in the target quadrant) during the probe trials of the water maze (r=-.81, p<.02).

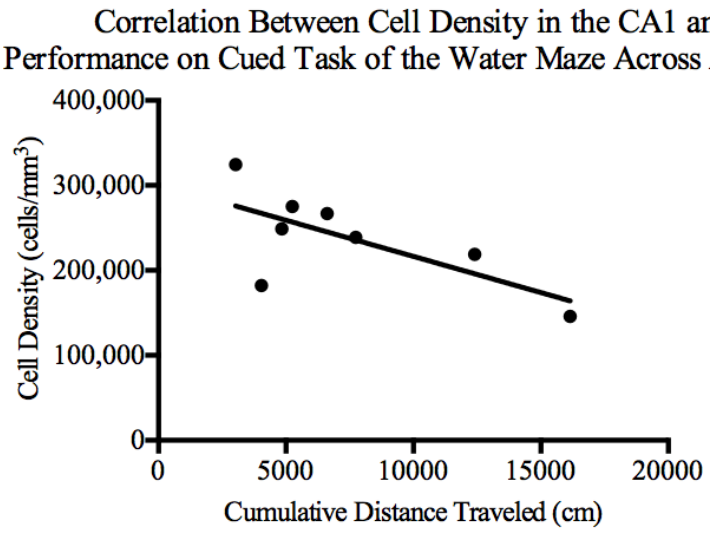

Figure 21. Across both groups of mice, lower cellular density in the CA1 was associated with worse performance (traveling a greater distance) in the cued task of the water maze (r=-.69, p<.059).

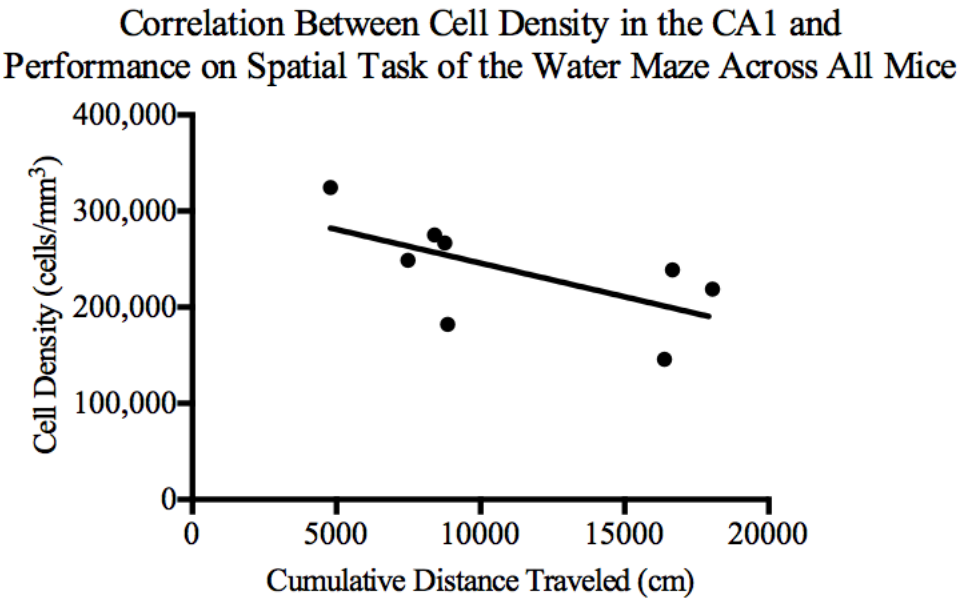

Figure 22. Across both groups of mice, lower cellular density in the CA1 was associated with worse performance (traveling a greater distance) in the spatial task of the water maze (r=-.63, p<.094).

Correlation analyses revealed no relationships between Aβ or amyloid load and cellular density in the CA1 and dorsal cortex. There were also no statistically significant correlations between fibrillar Aβ and total Aβ.

Repeated measures analyses suggested that PDAPP:E4 mice had more severe spatial learning deficits than PDAPP:E3 mice (Figure 23), as corroborated by Figure 22. PDAPP:E4 mice also spent more time in the open quadrants of the zero maze than PDAPP:E3 mice ($F(1,7)=5.99$, $p<.045$; Figure 24).

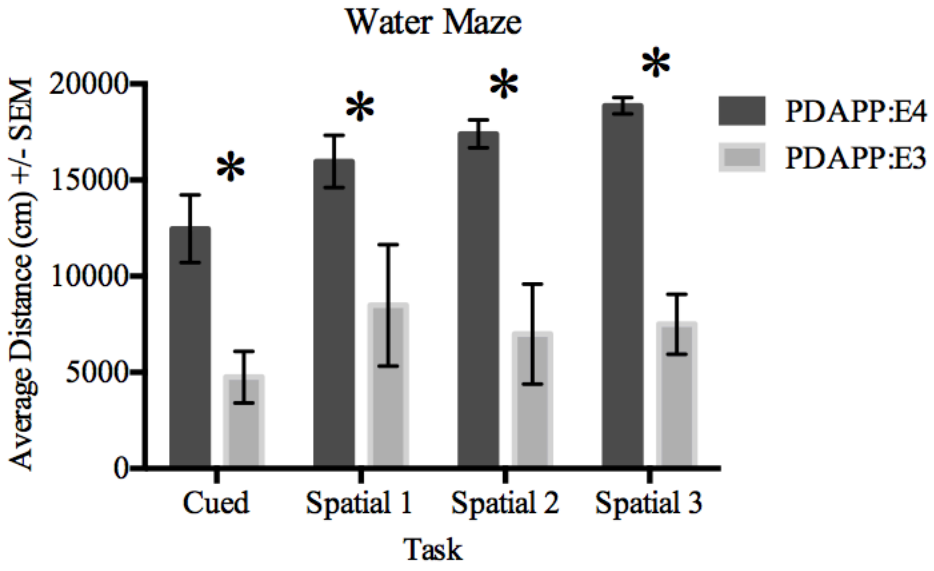

Figure 23. Repeated measures analyses suggested that PDAPP:E4 mice had worse spatial learning performance than PDAPP:E3 mice.

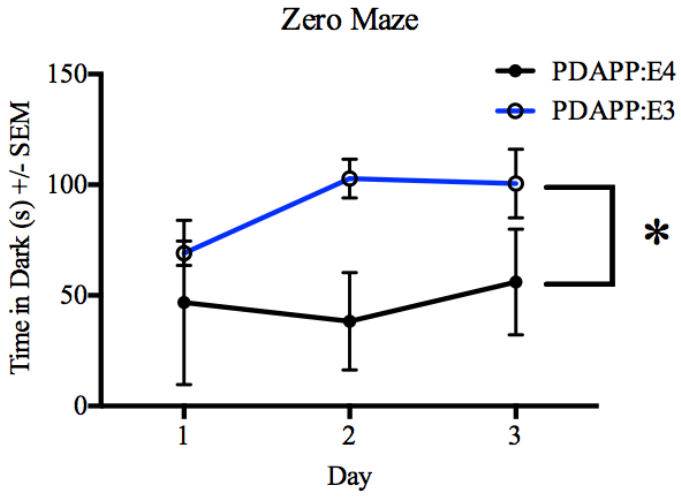

Figure 24. PDAPP:E4 mice spent more time in the open quadrants (less time in the dark) in the zero maze compared to PDAPP:E3 mice (p<.05).

To summarize the results of Hypothesis 3, PDAPP mice expressing E4 had worse behavioral deficits than PDAPP mice expressing E3, which correlated with their Alzheimer's-like neuropathology. In both sets of mice, those with more Alzheimer's-like neuropathology (specifically Aβ plaques and/or lower cellular density) had worse behavioral deficits.

CHAPTER SIX

DISCUSSION

The current study was designed to test the hypothesis that PDAPP:E4 mice would have greater neuropathology (including greater Aβ plaque load and lower cellular density in the brain) than PDAPP:E3 mice. Based on previous literature, the presence of E4 leads to greater Aβ production than does E3 in transgenic mice and humans (Bales et al., 1999, 2009; Mahley, Weisgraber, & Huang, 2006; Schmechel et al., 1993; Tiraboschi et al., 2004; Ye et al., 2005). Also, Hartman et al. (2002) concluded that in the presence of traumatic brain injury, fibrillar Aβ was only present in the PDAPP:E4 mice and not the PDAPP:E3 mice.

Previous studies have shown that Aβ aggregation induces cell death (Estus et al., 1997; LaFerla, Hall, Ngo, & Jay, 1996; Loo et al., 1993). Hippocampal cell loss has also been reported in AD patients (Mann, 1996; West, Coleman, Flood, & Troncoso, 1994). Hippocampal cell loss has also been reported in animal models such as in *in vitro* rat (Estus et al., 1997) and mouse (Ji et al., 2002; Loo et al., 1993) AD models as well as in *in vivo* mouse (Casas et al., 2004; LaFerla et al., 1996; Schmitz et al., 2004) AD models.

Our findings indicate that PDAPP:E4 brains had more total Aβ (diffuse and fibrillar Aβ) in the hippocampus and dorsal cortex than PDAPP:E3 brains, and that the difference was greatest in the dorsal cortex. These findings were corroborated by the fibrillar Aβ:total Aβ ratio analysis (Table 3). There was no statistically significant difference in fibrillar Aβ between PDAPP:E4 and PDAPP:E3 brains, but a trend suggests that PDAPP:E4 brains had less fibrillar Aβ in the hippocampus and dorsal cortex than PDAPP:E3 brains.

Findings also indicated no difference in cellular density between PDAPP:E4 and PDAPP:E3 brains in the CA1 or dorsal cortex. However, a trend was observed such that PDAPP:E4 brains had a lower cellular density than PDAPP:E3 brains in the CA1 and dorsal cortex.

Years of evidence have suggested that Aβ accumulation in the brain is the main causal factor of AD (Kim et al., 2011; Kuszczyk et al., 2013). One potential explanation for the trend that PDAPP:E4 brains had less fibrillar Aβ in the hippocampus and dorsal cortex than PDAPP:E3 brains is that Aβ accumulation may present as part of an adaptive response to chronic brain stress such as oxidation by free radicals, metabolic dysregulation, and/or genetic factors. Some studies have even suggested that Aβ is important for healthy brain functions, and that the removal of Aβ can interfere with brain homeostasis (Castello & Soriano, 2013; Castello, Jeppson, & Soriano, 2014).

Another potential explanation for the trend found in the current study is that amyloid accumulation may serve as a neuroprotectant (Castellani et al., 2009). Castellani et al. (2009) suggests that Aβ is not responsible for the clinical manifestation of AD since it may protect cells from toxic intermediates. They concluded that since amyloid accumulation is a byproduct of upstream pathogenic events, it protects cells from the toxic protofibrils (transient structures during the formation of mature amyloid fibrils) or oligomers. In other words, the fibrils may be a better alternative to more neurotoxic smaller aggregates.

The fibrillar Aβ:total Aβ ratio analysis trended toward a lower ratio in PDAPP:E4 mice than in PDAPP:E3 mice, meaning that PDAPP:E4 mice had a lower proportion of fibrillar Aβ in the total Aβ load than PDAPP:E3 mice. These findings contradict the idea

that the presence of the apoe4 allele should lead to a greater Aβ accumulation than the presence of the E3 allele (Ji et al., 2002). However, these data may support the idea that amyloid accumulation serves a protective role in the brain. Since the E4 allele is associated with more neurotoxicity than the E3 allele, they serve less as a neuroprotectant. However, it is possible that the HJ3.4 stain was not potent enough or that the thioflavin-S staining protocol yielded too much background staining.

The current study also tested the hypothesis that mice with more Alzheimer's-like neuropathology (specifically Aβ plaques and/or lower cellular density) will have worse behavioral deficits. Hartman et al. (2005) found that Aβ plaques displayed in PDAPP mice contributed to behavioral deficits. Similarly, G. Chen et al. (2000) found a negative correlation between spatial learning capacity and plaque burden for PDAPP mice.

The current study found that PDAPP:E4 mice had worse behavioral deficits than PDAPP:E3 mice, and that these deficits were positively correlated with the degree of neuropathology. Several studies corroborate our findings such that amyloid load in mice is correlated with greater deficits in spatial learning tasks (G. Chen et al., 2000, Hartman et al., 2005, Hartman et al., 2006). Hartman et al., 2005 showed that these spatial learning deficits were ameliorated after treating transgenic mice with an antibody specific for human Aβ. Hartman et al., 2006 also showed that dietary supplementation with pomegranate juice decreased amyloid load and improve behavioral performance in transgenic mice.

For example, higher amyloid plaque loads in the dorsal cortex of PDAPP:E3 mice were associated with lower activity levels (distance moved [Figure 17] and duration spent moving [Figure 18]) in the open field task. PDAPP:E3 mice also showed lower activity

levels in the zero maze, spending a lot of time in the dark. Although PDAPP:E3 mice spent more time in the dark than PDAPP:E4 mice, PDAPP:E3 still spent a relatively lower duration of time in the dark compared to control wild type mice who spend about 2/3^{rds} of that time in the dark.

The current study also found that higher total Aβ loads in the hippocampi of PDAPP:E3 mice were correlated with better performance on the rotarod rotating at a steady rate, and higher total Aβ loads in the dorsal cortices of PDAPP:E4 mice were correlated with worse performance on the rotarod rotating at an accelerated rate. Contradictory to previous literature, one possible explanation for these results may be that Aβ accumulation may serve as a neuroprotective factor (e.g., by protecting cells from toxic protofibrils or oligomers) up until a substantial amount of balance and sensorimotor coordination is required (balancing on a rod rotating at an accelerated rate) in which case behavior declines, as observed in the findings.

Correlation analyses between cell count and behavioral deficits confirmed that mice with more Alzheimer's-like neuropathology have more behavioral deficits. Specifically, the degree of lower cellular density was positively correlated with deficits in spatial learning and memory. Similar findings were reported by Morris & Price (2001), in which they observed substantial cell loss in humans with mild AD. Multiple other studies then found that those with AD have behavioral deficits (Chen et al., 2000; Hartman et al., 2005), providing evidence for the positive correlation between cell loss and behavioral deficits.

PDAPP:E4 mice had more spatial learning deficits, spent more time in the open quadrants of the zero maze, and had worse performance on the rotarod than PDAPP:E3

mice. These findings were likely due to motor deficits and/or abnormal exploratory/risk-taking behaviors.

Limitations

One main limitation of the current study is the low sample size. Many animals could not be included in the histological analysis due to high levels of old-age related mortality that often occurred overnight, rendering the brains difficult or impossible to process. This, in addition to the exclusion of outliers, yielded a low sample size that likely played a strong influence on high degree of variance observed. Further research is encouraged with a larger sample size.

Conclusion

The hypotheses for the current study, based on clinical observations in humans, was that PDAPP:E4 mice would have more Aβ plaques and lower cellular density in the CA1 and dorsal cortex than PDAPP:E3 mice, and that higher levels of neuropathology would be correlated with worse behavioral deficits.

The findings from the current study partially corroborated those from Bales et al., 1999 & 2009 in that diffuse Aβ was significantly greater in PDAPP:E4 mice than in PDAPP:E3 mice in the hippocampus and cortex. Although Bales et al., 1999 & 2009 also reported significant differences in fibrillar Aβ, the difference was not significant in the current study. The current study's novel findings include significantly worse behavioral performance in the PDAPP:E4 mice, as well as a trend for PDAPP:E4 mice to have fewer

cells per volume in both the hippocampus and cortex. Mice with more Alzheimer's-like neuropathology generally had worse behavioral deficits.

As illustrated in Table 5, various animal models for AD have been studied. The animal model from the current study (second to last row; Table 5) is the exact same animal model as the one studied in Bales et al., 1999 & 2009 (third to last row; Table 5.)

The PDAPP:E mice in this study replicate several features of Alzheimer's disease (last row; Table 5), and may therefore be a good model for studying how apoE genotype affects the development of the disease's neuropathological and behavioral sequelae and its response to therapeutic treatments, especially those designed to reduce plaque accumulation.

Table 5. Summary of findings of various types of AD models and how they compare to the findings of the current study.

Model	Study	CA1			Dorsal Cortex			Tau	Beh. Deficits
		Diff. Aβ	Fibr. Aβ	Lower Cell Density	Diff. Aβ	Fibr. Aβ	Lower Cell Density		
Humans with AD	A	✓	✓	✓	✓	✓	✓	✓	✓
PDAPP	B	✓	✓	✓	✓	✓	✗	✓	✓
APP23, APP751, Tg2576, APP695, TG Mice	C	✓	✓	✓	✓	✓	✗	✓	✓
Human E4 Mice	D	✓	✓	✗	✓	✓	✗	✓	✓
PSAPP, TASTPM Mice	E	✗	✓	✓	✗	✓	✓	✓	✓
PDAPP:E4 Mice	F	✓	✓	✗	✗	✗	Trend	N/A	Trend
PDAPP:E4 Mice (KI)	G	✓	✓	N/A	✓	✓	N/A	N/A	N/A
Current Model: PDAPP:E4 Mice	H	✓	✗	Trend	✓	✗	Trend	N/A	✓
Combined Model	I	✓	✓	Trend	✓	✓	Trend	N/A	✓

Note. "Diff." = diffuse, "Fibr." = fibrillar, "Beh." = Behavioral, ✓= Present, ✗= Absent, N/A = Not Tested; A. Bookheimer et al. 2000, Braak & Braak, 1995, Burggren et al., 2008, Corder et al., 1993, & Schmechel et al., 1993; B. Duyckaerts et al., 2007, Games et al., 1995; German et al., 2005; Holtzman et al., 2000; C. Calhoun et al., 1998, Hartman et al., 2006, Hsiao et al., 1996, & Schmitz et al., 2004, Tomidokoro et al., 2001; D. Buttini et al., 2002, Grootendorst et al., 2005, Raber et al., 2004, Tesseur et al., 2000, & Tiraboschi et al., 2004; E. Holcomb et al., 1998, Howlett et al., 2008, & Takeuchi et al., 2000; F. Fagan et al., 2002 & Hartman et al., 2002; G. Bales et al., 1999 & Bales et al., 2009; H. Current study; I. Bales et al., 1999, Bales et al., 2009, & Current study.

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