USING APOE GENOTYPES TO IDENTIFY NEW BIOMARKERS FOR ALZHEIMER’S DISEASE RISK

A Dissertation
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Graduate School of Arts and Sciences
of Georgetown University
in partial fulfillment of the requirements for the
degree of
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in Neuroscience

By

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Alzheimer’s disease (AD), unlike the other leading causes of death, does not have a cure or an effective intervention strategy. The largest genetic risk factor for AD is APOE, with the ε4 allele increasing and the ε2 allele decreasing one’s risk for the disease. It remains unclear how ApoE isoforms contribute to various AD-related pathological changes (e.g. amyloid plaques, synaptic and neuron loss). Here, we characterize the differences between the at risk group for AD (the ε4 carriers) and the not-at risk group (non-ε4 carriers), to determine what underlies APOE-related risk to AD.

To do this, we utilized APOE Targeted Replacement (TR) mice. These animals express the human APOE alleles (APOE−ε2, APOE−ε3, or APOE−ε4) under the mouse APOE promoter, and do not develop the plaques and tangles diagnostic of AD. We found that despite the lack of AD pathology, APOE-ε4 TR mice had alterations at the synapse. Specifically, APOE-ε4 TR mice have fewer dendritic spines at the post-synaptic terminal and simpler neuronal morphology compared to the other APOE genotypes. Pre-synaptically, we found that APOE-ε4 TR mice have reduced levels of glutaminase, increased levels of VGLUT1 and increased levels of glutamine (GLN). Taken together, these data suggest that the APOE-ε4 allele affects brain function well before AD pathogenesis occurs.
To begin addressing the mechanism by which APOE can impact dendritic spine morphology, we examined the role of the apoE receptor, ApoEr2. We found that increased surface levels of ApoEr2 promoted dendritic spine formation and that the ligand binding domain is necessary for us to observe these effects, suggesting that ApoEr2 may be involved in APOE related changes at the synapse.

To test whether there are CSF biomarkers of APOE-associated risk that could be followed in preventative therapeutic AD approaches, we examined levels of GLN in ante-mortem CSF samples from healthy controls. Consistent with our mouse studies, we found that APOE-ε4 carriers had higher levels of GLN compared to the other genotypes. These studies suggest that GLN may be a novel biomarker used to assess AD patients in their pre-clinical phases and as a therapeutic measure in preventative AD trials.
The research and writing of this thesis
is dedicated to everyone who helped along the way.

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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid beta</td>
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<tr>
<td>AO</td>
<td>Apical oblique</td>
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<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
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<tr>
<td>APOE</td>
<td>Apolipoprotein E (gene)</td>
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<tr>
<td>ApoE</td>
<td>Apolipoprotein E (protein)</td>
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<tr>
<td>ApoEr2</td>
<td>ApoE receptor 2</td>
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<tr>
<td>BS</td>
<td>Basal shaft</td>
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<tr>
<td>CSF</td>
<td>Cerebral spinal fluid</td>
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<tr>
<td>Cho</td>
<td>Choline</td>
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<tr>
<td>CDR</td>
<td>Clinical dementia rating</td>
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<tr>
<td>Cr</td>
<td>Creatine</td>
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<tr>
<td>DHA</td>
<td>Dehydroascorbic acid</td>
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<tr>
<td>DIV</td>
<td>Days in vitro</td>
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<tr>
<td>EAAT</td>
<td>Excitatory Amino Acid Transporter</td>
</tr>
<tr>
<td>FAD</td>
<td>Familial AD</td>
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<tr>
<td>FDG</td>
<td>Fluorodeoxyglucose</td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
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<td>Glutamate</td>
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<td>GLN</td>
<td>Glutamine</td>
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<tr>
<td>HE</td>
<td>Hepatic encephalopathy</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>LC-MS</td>
<td>Liquid-Chromatography- Mass Spectrometry</td>
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<tr>
<td>LTP</td>
<td>Long term potentiation</td>
</tr>
<tr>
<td>MRS</td>
<td>Magnetic Resonance Spectroscopy</td>
</tr>
<tr>
<td>mEPSC</td>
<td>Mini Excitatory Post Synaptic Currents</td>
</tr>
<tr>
<td>MSO</td>
<td>Methionine sulfoximine</td>
</tr>
<tr>
<td>mIns</td>
<td>Myoinositol</td>
</tr>
<tr>
<td>NAA</td>
<td>N-acetylaspartate</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-Steroid Anti-Inflammatory</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PAG</td>
<td>Phosphate-activated glutaminase</td>
</tr>
<tr>
<td>Tau</td>
<td>Taurine</td>
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<tr>
<td>TR</td>
<td>Targeted Replacement</td>
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<tr>
<td>VGLUT1</td>
<td>Vesicular glutamate transporter 1</td>
</tr>
<tr>
<td>VLDLR</td>
<td>Very low density lipoprotein receptor</td>
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CHAPTER I: INTRODUCTION

A. Alzheimer’s disease

Alzheimer's disease (AD), is a common source of disability in the elderly. It accounts for 60-80% of dementia cases and by 2050, it will affect 1 in 85 people worldwide (1). AD is an age-related neurodegenerative disease that is characterized by progressive memory loss and deteriorating cognitive function (2). Pathophysiologically, AD is characterized by extracellular amyloid beta (Aβ) plaques, intracellular hyper-phosphorylated tau tangles, inflammation, gliosis, and brain atrophy resulting from synaptic and neuronal loss (3).

The Amyloid Cascade Hypothesis

The prevailing theory for AD pathogenesis is the Amyloid Cascade Hypothesis. This hypothesis posits that Aβ deposition is the initiating sequence in the cascade of events that result in subsequent Aβ plaque formation, tau hyper-phosphorylation, inflammation, and synaptic loss eventually resulting in cell death (4). Aβ is the cleavage product of a type I membrane protein, the amyloid precursor protein (APP). Evidence that Aβ is the initiating factor in AD pathogenesis came from work with familial AD (FAD) patients. The majority of FAD cases have mutations in APP or in Presenilin 1 or 2, a gamma secretase subunit involved in Aβ cleavage formation. These mutations impact Aβ production or the facility of Aβ to aggregate, indicating that Aβ is the primary event for the resulting AD pathology (4-5). Moreover, Down syndrome
individuals, who have three copies of chromosome 21, where the APP gene is located, also
develop clinical and pathological signs of early onset AD (6).

A recent study examining cerebral spinal fluid (CSF) and magnetic resonance imaging
from FAD and control patients found that pathio-physical changes occurred 2 decades before
clinical diagnosis (7). In line with the amyloid cascade hypothesis, Bateman et al. report that in
FAD patients, CSF Aβ levels were significantly different from controls beginning at 20 years
before disease onset, increases in CSF Tau were significantly increased 15 years before onset
and mild cognitive impairments were only observed 5 years before diagnosis. These findings
suggest that the diagnosis of AD is made late in the course of the biologic cascade of disease
progression.

Sporadic AD, which has no clear genetic cause, is the predominant form of AD. The
amyloid cascade posits that it occurs due to an accelerated age-dependent Aβ accumulation. At
some point, when sufficient Aβ in an oligomeric form has accumulated, the amyloid cascade for
sporadic AD ensues (4). If the sporadic AD also has a “prodromal” period, individuals would be
outwardly asymptomatic, but would have already begun the amyloid pathogenic cascade.
Therefore there is a need to identify biomarkers of the strong risk of AD before pathogenesis,
since altering AD progress at later stages is very difficult (8). Well-validated biomarkers for AD
can be used in developing more effective therapeutics, prevention trials and improve the design
of clinical trials.

Current Biomarkers used for Alzheimer’s Disease
Ideally, biomarkers are reliable predictors of the disease progression. There are current efforts to find such markers for AD in neuroimaging and in protein levels in CSF and blood. For neuroimaging, observations suggest that prior to AD, there is shrinkage in the hippocampi as evidenced by MRI studies (7, 9-10) and reduced glucose uptake as evidenced by fluorodeoxyglucose (FDG)-PET signal in parietal and posterior cingulate brain regions compared to controls (11-13). However, there is no clear agreement about how much hippocampal shrinkage and FDG-PET signal reduction is indicative of a disease process in the population at large.

This past year, the FDA approved use of Amyvid (generic name Florbetapir F18), an amyloid imaging contrast agent which can be used in PET scans to detect amyloid binding. Studies have shown that Amyvid staining is increased in subsets of mild cognitively impaired patients, as well as in AD patients, and that these levels correlate with autopsy levels (14-15). This FDA approval, however, is not for predicting AD development, but for ruling out other diseases. For example, if a patient has cognitive impairment but little Amyvid binding, this would suggest that the patient does not have AD but another dementia.

Abnormal measurements of Aβ and Tau are observed in CSF for those in the early stages of AD (16). As discussed above, changes to Aβ and Tau in CSF can begin occurring two decades before the actual disease diagnosis in FAD cases (7). Interestingly, Aβ levels are reduced while Tau levels are increased in early stages of AD. The reduction in Aβ is believed to be due to reduced clearance of Aβ from the brain to the CSF, resulting in more brain Aβ deposits, which forms plaques. Although Tau is a cytoskeleton protein, and is not secreted, the increases in Tau are thought to be due to initial neuronal cell death, which would release Tau to the extracellular
space for clearance. Actual levels measured vary from institution to institution, creating challenges in interpreting what levels imply a disease state. Therefore, current quality controls are being put in place, such as standardized protocols, to overcome this obstacle and begin quantifying levels as a potential indicator (16).

Risk Factors for Alzheimer’s Disease

The largest overall risk factor for AD is age, with one’s risk of being diagnosed with AD exponentially increasing starting at 65 years of age (2). The largest genetic risk factor for late-onset AD is apolipoprotein E (APOE), which encodes for a protein responsible for cholesterol and lipid transport (17-19). Other genetic risk factors have been reported to affect AD risk such as Apolipoprotein J (also known as clusterin), the synaptic adhesion molecule PICALM, and the inflammatory markers CD1 and TREM2 (20-22). However, these other risk factors contribute only a small amount to the risk of AD compared to APOE, which can increase one’s risk by 300% (18).

B. Apolipoprotein E

APOE Genotype and Alzheimer’s Disease

Unlike murine ApoE, humans have three different isoforms of ApoE: ε2, ε3, and ε4, which differ by single amino acid substitutions at the 112th and 158th positions. The ε4 allele
increases the risk for developing AD by 300% (17-18) while the ε2 allele decreases this risk by 40% (23). Interestingly, APOE genotype affects the age of onset but not the rate of progression of AD, with ε4 carriers getting AD at earlier ages compared to non-ε4 carriers (19). These observations have lead to the hypothesis that APOE affects an early process in AD pathogenesis such as Aβ deposition and clearance.

Evidence supporting this hypothesis include experiments showing that APOE genotype affects the aggregation of Aβ in vitro and levels of extracellular Aβ in human and transgenic mouse brain (for review, see (24)). For example, in vitro binding studies show that lipid-free ApoE-ε4 binds to Aβ with a higher affinity than apoE-ε3 (25-26) and induces aggregation (27-28). Transgenic mouse studies show that APOE isoforms differentially regulate the clearance of brain Aβ. For example, in vivo brain microdialysis of mice expressing human APOE isoforms found that the clearance rate of Aβ as APOE dependent with E4 < E3 < E2 (29). Moreover, APOE-ε4 mice have earlier and more severe pathology than those expressing APOE-ε3 (30-31). Similarly, human APOE-ε4 carriers with AD have higher levels of Aβ plaque deposition (32) and increased PIB retention (amyloid imaging) compared to APOE-ε4 negative carriers (33).

**APOE Targeted Replacement Mice**

To assess APOE-ε4 related changes in brain function, we and others have utilized APOE Targeted Replacement (TR) mice. These animals express the human APOE alleles (APOE-ε2,
APOE-ε3, or APOE-ε4) under the mouse APOE promoter, and do not develop the plaques and tangles diagnostic of AD (34). Despite the lack of neuropathological changes, APOE-ε4 TR mice have simpler neuronal structures in the amygdala (35) and abnormalities in hippocampal long term potentiation (LTP), (36-37), and alterations in hippocampal structure (38). The APOE-ε4 animals have behavioral deficits in some Morris Water Maze tasks such as the probe test but not the learning rates while training (38-40). Moreover, aged female APOE-ε4 TR mice display increased errors in avoidance conditioning tasks that are not observed in younger cohorts (39-40). Interestingly, a recent study has reported an emergence of a seizure phenotype in aged APOE-ε4 TR mice and abnormal cortical EEG activity (41). These studies support the human studies described above, which suggest that APOE genotype affects brain structure and activity in the absence of overt signs of AD.

*Apolipoprotein Structure*

ApoE is a secreted 299 amino acid protein, and in the brain, apoE is predominantly synthesized by astrocytes and microglial cells. ApoE is the main component of lipoproteins, whose primary function is to bring lipids to cells. The lipid binding domain of apoE, amino acids 244-272, have amphipathic alpha-helical structures, which allow apoE to easily switch between being lipid bound and lipid free (42). The N-terminus is comprised of 4 helical structures, where amino acids 136-150 (on the 4th helix) comprise the apoE-receptor binding region. As noted above, apoE is polymorphic, with the three most common alleles (ε2, ε3 and ε4), differing at
position 112 and 158. ApoE3 is the most common isoform, containing a cysteine at the 112 position and an arginine at the 158 position. In contrast, apoE2 has two cysteines and apoE4 has two arginines at these positions. Therefore, apoE4 is the only apoE with an arginine at the 112 position and it is this Arg112, which alters the domain interaction between the N- and C-terminal of apoE (42).

Domain Interactions of ApoE

The 112 residue of apoE affects the conformation of the side chain of Arg61. In apoE4, the Arg112 orients the Arg61 side chain away from the N-terminus (43). This orientation enables the positively charged Arg61 to interact with the negatively charged Glu255 in the C-terminus (44). In apoE3 and apoE2, the Arg61 side chain remains oriented towards the other α-helices in the N-terminus and therefore the Arg61 does not interact with the C-terminus. This has been confirmed using X-ray crystallography (44) and with in vitro FRET studies expressing apoE with a GFP tag on the N terminus and a YFP tag on the C terminus (45). These domain interactions are posited to allow for easier fragmentation of apoE, which may contribute to neurotoxicity (46).

Interestingly, humans are the only species that have an apoE with Arg61. All other species have a threonine at this position. A point mutation in human apoE4 from Arg61 to a threonine eliminates the N- and C-terminal domain interaction. Therefore, although murine apoE has an Arg112 like apoE4, it behaves more like an apoE3 in its tertiary form (47).
ApoE4 May Have Reduced Stability

The domain interaction observed in apoE4, allows it to form a molten globule like structure which is linked to reduced stability (48). Indeed apoE4 is least resistant to denaturation (46, 49). Moreover, APOE-ε4 knock-in mice have lower levels of ApoE protein observed in brain and plasma compared to APOE-ε3 knock-in mice, despite having similar transcription rates (50-51). A recent study has found that healthy APOE-ε4 human carriers also have reduced levels of apoE in their CSF (29), giving further credence to apoE4 being more easily degraded. These observations have led to a debate about whether apoE4 effects are due to loss of function (through enhanced degredation) or gain of function (by enhanced toxicity through its altered tertiary structure).

ApoE Receptors

ApoE receptors are a family of transmembrane proteins that mediate endocytosis of ligands and are then recycled back to the cell surface (52). ApoE receptors include the LDL receptor, LDL receptor related proteins (LRP-1, LRP-1B, LRP-2), ApoE receptor 2 (ApoEr2), and the very low density lipoprotein receptor (VLDLr). Each of these type I transmembrane receptors has a large N-terminal extracellular domain, with multiple ligand-binding repeats, and small C-terminal cytoplasmic adaptor domains with one or several NPXY sequences for receptor-mediated endocytosis. These ApoE receptors are involved in neuronal migration during brain development (53), influx of calcium through NMDA channels (54), neurite outgrowth (55),
long term potentiation (LTP) and memory (56). However, the mechanisms by which ApoE receptors affect LTP, learning, and memory are unclear.

*ApoE Receptor 2 (ApoEr2) and its Cytoplasmic Adaptor Proteins Involved in Synaptic Plasticity*

ApoE receptors interact with cytoplasmic adaptor proteins via specific binding motifs. One of these receptors, ApoEr2, can interact with synaptic cytoplasmic adaptor proteins such as PSD-95 (56-59), a major postsynaptic density protein important for synapse formation and function (60), through a domain encoded by the alternatively spliced ApoEr2 exon 19 (58). This region of ApoEr2 regulates memory and behavior in mice (56). In addition, proteins in the X11 family also interact with ApoEr2 via exon 19 (61-62). X11 family members (X11α, β and γ, also referred to as mint-1, -2, and -3 for munc interaction) are present at both presynaptic and postsynaptic membranes (63). Presynaptically, X11α plays essential roles in vesicle docking and exocytosis via interactions with munc and CASK:Veli (64-65). X11α is also involved in synapse formation and neuroligation (65-66). However, it is unclear how interactions between ApoEr2 and its cytoplasmic adaptor proteins are involved in synapse and dendritic spine formation. Taken together, the data suggest that ApoEr2 is intimately involved in synaptic regulation, implying that its ligand, ApoE, may also be involved in synaptic function.

*APOE and Normal Brain Function*
Due to the multiple roles that apoE can have via its receptor mediated signaling or through delivery of cholesterol, it is not surprising that APOE genotype affects normal brain function independent of AD pathology, as evidenced by APOE TR mice (see section above on pg 6) as well as by studies of young, non-demented humans (67-69). Specifically, in humans, the ε4 allele of APOE is associated with deficits in glucose metabolism in cognitively unimpaired individuals in their 50s-60s (70-72), and with alterations in cerebral activation (measured by PET or fMRI) in young adults (67-69). These findings suggest that APOE’s impact on brain function early in life could contribute to the susceptibility to damages later in life. Consistent with this idea, APOE genotype affects outcomes of other conditions of brain damage besides AD, including traumatic brain injury (73-74), HIV-dementia (Burt et al., 2008), and stroke (75). APOE also affects cognitive decline in subjects with non-AD dementias (76). These observations support the development of a new class of therapeutics to compensate for APOE-ε4 related changes prior to overt signs of disease. If we can characterize the APOE-ε4 related changes which increase one’s risk towards AD, we could then extend this to the whole population to assess whether these characterizations could be used as a generalization to the population at large.

C. Outline of Topics Addressed in Thesis

ApoE is implicated in a variety of mechanisms in the central nervous system ranging from neuronal development (77-79) to brain plasticity and repair (80-81). APOE genotype may also have a profound influence on the extent of disease-related synaptic deterioration due to its effects
on dendritic growth: apoE3 promotes, while apoE4 reduces, neuritic outgrowth in vitro (77-78, 82). In vivo and in vitro studies have also exhibited a role for apoE4 in reducing neurite sprouting (35, 83). Together, these findings led us to hypothesize that apoE4 may impede dendritic outgrowth and decrease the formation of new synapses, thereby contributing to AD development.

To test this hypothesis (Chapter III), we systematically examined the effect of all three apoE isoforms on dendritic spine number (sites of post-synaptic excitatory neurotransmission) in cortical layers II/III and in the dentate gyrus of mouse brains. Using APOE TR mice, we found that at all ages, APOE-ε4 TR mice had decreased spine density in cortical neurons compared to APOE-ε2 and APOE-ε3 TR mice but not in dentate gyrus granule cells. Furthermore, APOE-ε2 TR mice had increased dendritic complexity compared to APOE-ε3 and APOE-ε4 TR mice. These results show that APOE isoforms differentially affect neurite and dendritic spine morphologies in vivo, suggesting these effects could modulate normal brain functions as well as reparative functions affecting risk, prognosis, and disease course in many CNS disorders.

To begin addressing the mechanism by which APOE can impact dendritic spines, we began by examining the ApoE receptor, ApoEr2, to test whether it could be a viable candidate for modulating synaptic plasticity (Chapter IV). Over-expression of ApoEr2 in primary cultures enhanced dendritic spine formation, and increased synaptic markers. In contrast, ApoEr2 deficient mice displayed reduced dendritic spine density in the cortex. Furthermore, the ligand binding domain of ApoEr2, where ApoE can bind, was necessary for this effect to be observed. These data suggest that apoEr2 may be one of the mechanisms through which apoE can impact dendritic spine formation.
Because we had found differences in the morphology of post-synaptic excitatory sites (ie. dendritic spines), we asked whether APOE genotype also affected normal excitatory neurotransmission at the synapse by examining the elements of the glutamate-glutamine cycle in the brain (Chapter V). APOE-ε4 TR mice had lower levels of glutaminase and higher levels of the VGLUT1 transporter. Moreover, we examined the production and metabolism of glutamate (GLU) and glutamine (GLN). Using high frequency $^{13}$C / $^1$H nuclear magnetic resonance (NMR), we found that incorporation of $^{13}$C label from glucose into C4 and/or C3 isotopomers of glutamate was decreased in APOE-ε4 TR mice. However, APOE-ε4 TR mice had higher levels of brain glutamine. Taken together, these data suggest that APOE genotype affects presynaptic terminal composition and impacts the normal GLU-GLN cycle.

Using transmission electron microscopy, we then assessed whether APOE genotype could also impact the ultrastructural components of an excitatory synapse, specifically the mitochondrial size and synaptic vesicle number (Chapter VI). We found that the surface area of mitochondria were significantly decreased in APOE-ε4 TR mice compared to APOE-ε3 TR mice, suggesting that mitochondrial efficiency specifically ATP and calcium buffering may be altered.

Our ultimate goal is to understand the differences in APOE genotype to uncover new biomarkers for AD risk. Therefore, based on our findings in Chapter III, we began to examine levels of glutamatergic subunits in mouse brain, in the hopes of uncovering a better assay for reduced dendritic spine density (Appendix A). Based on chapter V and VI, which hinted at potential mitochondrial deficiencies, we also utilized $^1$H-MRS in our APOE TR mice, to assess potential differences in energetics in a non-invasive way (Appendix B). Based on chapter V,
where we found that total levels of GLN being significantly increased in APOE-\(\varepsilon\)4 TR mice compared to the other genotypes, we conducted a preliminary study, using liquid-chromatography mass spectroscopy to quantitatively determine GLN levels in ante-mortem CSF from cognitively healthy controls (Chapter VII). We found that \(\varepsilon\)4 carriers had increased GLN levels, suggesting that this may be a valid biomarker to use for increased AD risk. Future experiments will extend on these findings, to assess the mechanism by which GLN levels are high, and whether high levels of GLN (even in non-\(\varepsilon\)4 carriers) increase one’s risk of getting AD.

D. Hypothesis

My hypothesis is that APOE genotype affects brain structure and function before AD pathogenesis, resulting in \(\varepsilon\)4 carriers being more vulnerable to the damages that occur with aging. If this is true, potential preventative therapeutics for AD could be developed to compensate for the APOE-\(\varepsilon\)4 related changes. To determine whether this is the case, I will characterize APOE knock-in mice that do not develop the plaques and tangles diagnostic of AD to determine whether any of the differences between the genotypes can be used as new biomarkers for the human population that is at risk for AD.
Chapter II: Methods

Mice:
For the majority of the experiments, human APOE2, APOE3 and APOE4 TR mice, which express each of the human APOE isoforms regulated by the endogenous murine APOE promoter (34-35) were used. These mice were initially donated by Dr. Patrick Sullivan (Duke University) after which, an in-house colony at Georgetown University was established. Briefly, all three lines of apoE TR mice contain chimeric genes consisting of mouse 5’ regulatory sequences continuous with the non-coding region (mouse exon 1) followed by human exons (and introns) 2–4 (34) Mice were backcrossed to C57BL/6J mice eight times and therefore are >99.6% C57Bl/6J before breeding at Georgetown began. APOE-deficient mice and C57BL/6J wild type mice were obtained from Jackson Laboratories (Bar Harbor, Maine, USA). For the ApoEr2 experiments, ApoEr2 null mice were raised from stocks originally created through targeted-deletion of each individual gene (84) at the University of Southern Florida under animal protocol number R3336. Wild type littermates were used as controls for all ApoEr2 null experiments. All animal experiments were conducted in compliance with the rules and regulations of the Institutional Animal Care and Use Committee at Georgetown University.

Golgi Staining and Analysis of Dendritic Morphology in vivo:
Golgi staining was performed on APOE2, APOE3, and APOE4 TR mice (4 weeks, 3 months and 1 year; \( n = 4 \) per genotype for each timepoint point), APOE knock-out mice (4 weeks and 3 months; \( n = 4 \) mice per genotype for each time point), and wild type C57BL/6J mice (1 year; \( n = \)
4 mice per genotype). For the ApoEr2 experiments, Golgi staining was performed on ApoEr2 knockout mice and wild type C57BL/6J littermate mice (4 weeks or 1 year old; n=4 per genotype at each time point). For all of these experiments, we used the FD Rapid Golgi Stain kit (FD NeuroTechnologies, Ellicott City, MD) as previously described (85). Briefly, freshly dissected brains were immersed in solution A and B for 2 weeks at room temperature and transferred into solution C for 24 h at 4°C. The brains were sliced using a Vibratome (VT1000S; Leica, Germany) at a thickness of 150 µm. Bright-field microscopy (Axioplan 2; Zeiss, Brighton, MI) images (at 63x magnification) were taken of pyramidal neurons in cortical layers II/III (56 neurons per genotype per time point), granule cells in the dentate gyrus (n=28 neurons per genotype per time point), and CA1 pyramidal neurons (n=56 neurons per genotype). Images were coded, and dendritic spines counted in a blinded fashion using Scion or Image J software (NIH, Bethesda, MD). All the spines counted were also measured for spine length as previously described (86).

Sholl Analysis:

For Ex Vivo Analysis: 18 pyramidal neurons in somatosensory cortical layers II/III were traced using Neurolucida Software (MicroBrightField, Williston, VA), for each group (APOE2, APOE3, and APOE4 TR mice) as previously described following golgi impregnation (85). Following the tracing, and setting the center of the soma as a focal point, we generated concentric circles around the center. The initial circle was of 20 µm radius and each circle increased the radius by 20 µm increments. We counted the number of intersections on the perimeter of each circle per neuron. For in vitro analysis: Primary hippocampal neurons were
transfected with GFP-B-actin and ApoEr2-HA or GFP-B-actin and empty vector at DIV 7. One week later, neurons were fixed in 4% paraformaldehyde and immunostained for GFP followed by DAB staining. Using Neurolucida software, neurons were traced and analyzed similarly as described above. A total of 10 neurons was used per condition in this analysis.

**Hippocampal Culture & Transfections:**

Primary hippocampal neurons from E18–E19 Sprague Dawley rats were cultured at 150 cells/mm² as described previously (86). For the ApoE experiments: primary hippocampal neurons (days in vitro (DIV) 12) were transfected with GFP and treated with 500 nM of recombinant apoE3 or apoE4 (Oxford Biomedical Research, Rochester Hills, MI, USA) or PBS as control for 48 hours. A total of 15 neurons from each group were analyzed. For the ApoEr2 experiments, neurons were transfected at 12, 14 or 16 DIV with GFP-ApoEr2, ApoEr2-HA, ApoEr2-myc, ApoEr2 deletion constructs with C-terminal myc tag, PSD-95-FLAG, X11α-FLAG, GFP or empty vector by lipofectamine 2000 (Invitrogen) (2 µg DNA per well) according to manufacturer’s instructions. All plasmids were driven by the CMV promoter.

**Sindbis Virus Infection:**

Primary hippocampal neurons were prepared as described above (87). Human ApoEr2 or GFP were cloned into the pSinRep5 Sindbis virus vector (Invitrogen) and replication-defective pseudovirions produced according to the manufacturer’s directions. Neurons were typically
between DIV14-16 at time of infection and the duration of infection was limited to 18-24 hr; no cytotoxicity was apparent during infection.

**COS-7 Cells and Transfections:**
COS-7 cells (Lombardi Co-Resources Cancer Center, Georgetown University) were maintained in Opti-MEM (Invitrogen) with 10% fetal bovine serum (FBS, Life Technologies, Inc.) in a 5% CO₂ incubator. The cells were transiently transfected with 0.5-1 µg of plasmid in FuGENE® 6 (Roche) according to the manufacturer’s protocol and cultured 24 hr in DMEM containing 10% FBS. For co-transfections, cells were similarly transfected with 0.5-1 µg of each plasmid in FuGENE® 6 (Roche) and cultured 24 hr in DMEM with 10% FBS.

**Preparation of Brain Homogenates:**
Whole brains were homogenized with a dounce homogenizer in ice-cold TBS buffer (50 mM Tris-HCl, 150 mM NaCl, 1x protease inhibitor and phosphate inhibitor cocktails, pH 7.4). The homogenates were centrifuged at 47,000 RPM for 45 min at 4°C and the supernatants were labeled as the TBS (soluble) fraction. The remaining pellet was sonicated in ice-cold TBS-X buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1x protease inhibitor and phosphate inhibitor cocktails, pH 7.4). The resuspended pellet was centrifuged at 47,000 RPM for 45 min at 4°C and the supernatants were labeled as the TBS-X (membrane) fraction. Otherwise, whole brains were homogenized with a dounce homogenizer in ice-cold RIPA (Sigma-Aldrich, St. Louis, Mo) buffer. The homogenates were sonicated and centrifuged at 13,000 RPM for 15
minutes. The lysate was labeled as RIPA fraction. Total protein concentration was determined by BCA protein assay kit (Pierce, Rockford, IL).

**Synaptosomal Fractionation:**

Synaptosome fractionation was performed as described (88), with minor modifications. Adult rat brains were briefly homogenized in 0.32 M sucrose, 4 mM HEPES-NaOH, pH 7.3 with protease inhibitors, and centrifuged at 1000 × g for 10 min to recover the supernatant S1 and the pellet P1. S1 fraction was centrifuged at 12,000 × g for 15 min to obtain the pellet P2 (crude synaptosome) and the supernatant S2. The P2 fraction was osmotically shocked by diluting with double-distilled water and further centrifuged at 25,000 × g for 20 min to generate the pellet LP1 and the supernatant LS1. The LS1 fraction was subjected to ultracentrifugation to obtain the synaptic vesicle fraction LP2. LP1 was detergent extracted in buffer B (0.16 M sucrose, 5 mM Tris-HCl, pH 8.0, 0.5% Triton X-100, 0.5 mM β-ME, 1 mM EDTA, and protease inhibitors), and then centrifuged at 33,000 × g for 20 min. The pellet LP1P was resuspended and applied to a discontinuous sucrose gradient consisting of 1.0 M, 1.5 M, and 2.0 M sucrose layers. After ultracentrifugation (200,000 × g for 2 hr), the PSD fraction was recovered at the interface between 1.5 and 2.0 M sucrose.

**Immunoblot Analysis:**

Protein samples were boiled in Laemmli buffer (4% SDS) and electrophoresed on 10% polyacrylamide gels with equal amounts of total protein loaded per lane. Separated proteins were transferred onto nitrocellulose membranes and analyzed by Western blotting. Membranes
were incubated with primary antibody for 1 hour at room temperature or overnight at 4°C followed by secondary antibody incubation. After incubation with the appropriate HRP-conjugated secondary antibody, membranes were developed using SuperSignal West PICO or DURA luminol/enhancer solution (Pierce). For negative controls, blots were analyzed in the absence of primary antibodies, to define endogenous mouse antibody bands by secondary antibodies. The X-ray film was scanned and the density of bands was quantified using Quantity One software (Biorad) or ImageJ.

**Immunohistochemistry:**

50 micrometer free-floating coronal sections were processed for peroxidase immunohistochemistry using rabbit anti-glutaminase (Abcam, 1:400). Tissue sections were incubated in biotinylated secondary antibodies, and detection of the peroxidase reaction product with diaminobenzidine was performed with the ABC Elite kit (Vector Laboratories, Burlingame, CA). Cresyl Violet was used as a counterstain for nuclei.

**Immunocytochemistry:**

Hippocampal cultured neurons were fixed either in 4% paraformaldehyde (for morphological analysis) or in methanol at –20°C for 10 min (for immunostaining of endogenous synaptic markers). Antibodies for immunostaining were incubated in GDB buffer (0.1% gelatin, 0.3% Triton X-100, 16 mM sodium phosphate pH 7.4, 450 mM NaCl). Cell surface expression levels of ApoEr2 were performed as described (89). Live neuronal cultures were briefly incubated (10 min) with antibodies directed against extracellular N-termi of GFP (10 µg/mL in conditioned
medium) to specifically label surface receptors, then lightly fixed for 5 min in 4% paraformaldehyde (non-permeabilizing conditions). After fixation, the surface-remaining antibody labeled GFP was measured with Alexa fluor 555–linked anti-mouse secondary antibodies for 1 hr. Immunostaining was quantified using Metamorph analysis of immunostaining intensity or punctate number from Z-stacked images obtained with a Zeiss LSM510 confocal microscope. Surface localization of staining was also confirmed visually from these images.

Antibodies:
The following primary antibodies from Abcam were used: rabbit anti-glutaminase, rabbit anti-glutamine synthetase, rabbit anti-EAAT1, rabbit anti-EAAT3, anti-HA, anti-c-myc. The following primary antibodies from Millipore were used: mouse anti-VGLUT1, rabbit anti-EAAT2, rabbit anti-GLUA1 and rabbit anti-GLUA2. The following primary antibodies from Sigma were used: rabbit anti-tubulin, mouse anti-β actin, anti-X11α/mint-1, anti-synaptophysin, anti-Flag. The following primary antibodies from BD Bioscience were used: The following antibodies from Chemicon were used: β-actin, anti-PSD-95, Anti-GFP came from Invitrogen and anti-X11α/mint-1 from BD Bioscience was also used. NDMA receptor subunit 2A and 2B were graciously donated by Dr. Bob Yasuda of Georgetown University.

High-frequency [1H/13C] Magnetic Resonance Spectroscopy:
Animals were fasted overnight with free access to tap water and were intraperitoneally (i.p.) injected with [1-13C] glucose solution (0.5 mol/l) over 10 s (0.3 ml/25–30 g body weight; 200
mg/kg). 45 min post-injection with [1,13C] glucose, animals were sacrificed by cervical
dislocation and brains immediately immersed in 6% ice-cold perchloric acid, 50 mM NaH2PO4.
This time point was chosen from previous studies where we had tested that 45 min was good
for 13C labeling of a range of metabolites in the mouse brain (90). After homogenization and
lyophilization, extracts were re-suspended in 0.65 ml D2O containing 2 mM sodium [13C]
formate as an internal intensity and chemical shift reference (δ 171.8). Metabolite pool size was
identified on 1H [13C-decoupled] NMR spectra. Peak areas were adjusted for nuclear Overhauser
effect, saturation and natural abundance effects and quantified by reference to [13C] formate.
Metabolite pool sizes were determined by integration of resonances in fully relaxed 400 MHz
[13C-decoupled] 1H spectra using N-acetylaspartate as internal intensity reference. Incorporation
of 13C into isotopomers was measured in reference to [13C] formate. All data were collected on a
9.7 Tesla Varian Spectrometer with dual 13C/1H probe. [13C-decoupled]-1H spectra were
acquired with 3000 scans, pulse width 45°, relaxation delay 1 s, line broadening 0.5 Hz,
acquired data points 13,132 and transformation size 32 K at room temperature. [1H-decoupled]-
13C spectra were acquired with 30,000 scans and 31,875 data points. Spectra were integrated and
quantified using MestReNova (Master Lab Research).

Transmission Electron Microscopy:

1 year old females (n=3/genotype) were perfused with 2.5% glutaraldehyde and fixed in 2.5%
glutaraldehyde overnight. The following day, brains were sectioned at 150 microns using
Vibratome (VT1000S; Leica), washed in 0.1 M sodium cacodylate buffer and then fixed in 2 %
osmium tetraoxide for 1 hour. The tissue was then dehydrated with increasing ethanol
concentrations starting at 50% then 75%, then 95%, then 100% (15 minutes, 2 times at each solution) and finally switched to propylene oxide for 30 minutes. Following dehydration, tissues were slowly embedded into plastic. Initially, tissue was transferred to a Propylene: EPON (AB), 1:1 dilution overnight at 4°C. EPON (AB) solution is: (EPON A: 62 ml EMBED 812 + 100 ml DDSA, EPON B: 100 ml EMBED 812 + 90 ml NMA; EPON AB: 13 ml of EPON A + 15 ml of EPON B + 16 drops of DMP-30). On the following day, tissue was transferred to a 3:1 EPON (AB): propylene oxide solution for 3 hours, and then a 100% EPON (AB) solution for 1 hour. Following this incubation, tissues were placed between two Aclar sheets (EMS) and placed into a 60°C oven for 72 hours. 72 hours, the top Aclar sheet was peeled off, so that the tissue was exposed to the air. Using a razor blade, a trapezoid section of the top part of the Dorsal cortex close to the midline was cut out and super glued to a pellet made of EPON. The second ACLAR layer was peeled off with a forcep under a microscope. Using a diamond blade, tissues were sectioned at 60-80 nm and placed on 200 mesh grids. Images were taken on a JEOL JEM-1200 EX Transmission electron microscope (Peabody, MA) equipped with an SIA-CAM, 4MP cooled CCD camera at 40,000 magnification. 120 (E3) and 140 (E4) images were taken for initial mitochondria experiments. 65 (E3) and 70 (E4) images were taken for mitochondria at synapses. 76 (E3) and 82 (E4) images were taken of pre-synaptic excitatory vesicles.

**Liquid Chromatography- Mass Spectrometry (LC-MS):**

We developed an LC-MS method to detect glutamate and glutamine, using standards from Cambridge Isotope Lab. We used 20 ul to prepare samples for analysis. Each sample was injected onto a HILIC 100 × 2.1 mm ACQUITY 1.7-μm column (Waters Corp, Milford, MA)
using an ACQUITY UPLC system (Waters). The column eluent was introduced directly into the mass spectrometer by electrospray. Mass spectrometry was performed on a Q-TOF Premier (Waters) operating in negative-ion or positive-ion electrospray ionization mode with a capillary voltage of 3.2 kV and a sampling cone voltage in negative or positive mode dependent on the metabolite. The desolvation gas flow was 1000 liters/h and the temperature was 500°C. Data were acquired in centroid mode from 50 to 400 \textit{m/z} in MSMS scanning, collision energy was optimized for each analyte to keep both the daughter and the parent ions in the spectrum for identification. For glutamate, the cone voltage was 18 V, the collision energy was 14 and 8, and the ion mode was positive. For glutamine, the cone voltage was 16 V, the collision energy was 10 and 16, and the ion mode was positive.

\textbf{\textsuperscript{1}H Proton Magnetic Resonance Spectroscopy (MRS):}

All of the magnetic resonance imaging was performed using the 7 Tesla Bruker horizontal bore Magnetic Resonance Imager run by Paravision 5.0 software which operates in the Lombardi Comprehensive Cancer Center’s Preclinical Imaging Research Laboratory. Prior to imaging, mice were anesthetized using 1.5% isoflurane and 30% nitrous oxide, positioned in a custom-made mouse stereotaxic device with temperature and respiration control, and imaged in a transmit volume coil in addition to a four channel receive array mouse brain coil. The MRS was performed as previously described (91). Briefly, single voxel proton MRS was performed using a volume-localized PRESS sequence with the following parameters: TE: 20 ms, TR: 2500 ms, averages: 1024, spectral width of 4 kHz, and 512 k complex data points and 6 Hz line broadening, using a voxel of 1-2 mm on edge. The voxel was positioned in the dorsal
hippocampus using a 3-D locator image as well as multi-plane images as required to correctly identify these regions in the three orthogonal planes. All in vivo peak integrated areas were analyzed by visual inspection using the using Bruker’s “TOPSPIN” or LC Model software in order to assess relative differences in tissue chemistry. The resolution of the spectra was defined as separation of the peaks at baseline. 7 animals per genotype (APOE-ε3 and APOE-ε4 TR mice) were imaged and analyzed in this manner.

Statistical Analysis:

Data are expressed as mean ± SEM unless otherwise specified. Statistical analysis was performed using the SPSS 10.0 software package (Graphpad, San Diego, CA). One-way analyses of variance (ANOVA) were used to analyze the effect of APOE genotype on various biomarkers of interest. Tukey's post-hoc analyses were used to detect statistical differences among the groups using *, p < 0.05, **, p<0.01, ***, p<0.001 compared to APOE-ε3 TR mice unless otherwise noted. A One Tailed Student’s T-Test was used for the CSF data (Chapter VII) Scholl analysis and spine density data was analyzed with GraphPad prism 4 software using one-way ANOVA followed by a Bonferroni post hoc test. For spine length data, the Kolmogorov-Smirnov statistical test was used.
Chapter III: ApoE4 decreases spine density and dendritic complexity in cortical neurons in vivo.\textsuperscript{a}

The three human alleles of apolipoprotein E (APOE) differentially influence outcome after CNS injury and affect one’s risk of developing Alzheimer’s disease (AD). It remains unclear how ApoE isoforms contribute to various AD-related pathological changes (e.g. amyloid plaques, synaptic and neuron loss). Here, we systematically examined whether apoE isoforms (\(\varepsilon2, \varepsilon3, \varepsilon4\)) exhibit differential effects on dendritic spine density and morphology in APOE targeted replacement (TR) mice which lack AD pathological changes. Using Golgi staining, we found age-dependent effects of APOE-\(\varepsilon4\) on spine density in the cortex. The APOE- \(\varepsilon4\) TR mice had significantly reduced spine density at three independent time points (4 weeks, 3 months, and 1 year, 27.7\% \pm 7.4\%, 24.4\% \pm 8.6\%, 55.6\% \pm 10.5\%, respectively) compared to APOE-\(\varepsilon3\) TR mice and APOE- \(\varepsilon2\) TR mice. Additionally, in APOE-\(\varepsilon4\) TR mice, shorter spines were evident compared to other APOE TR mice at 1 year. APOE-\(\varepsilon2\) TR mice exhibited longer spines as well as significantly increased apical dendritic arborization in the cortex compared to APOE-\(\varepsilon4\) and APOE-\(\varepsilon3\) TR mice at 4 weeks. However, there were no differences in spine density across APOE genotypes in hippocampus. These findings demonstrate that apoE isoforms differentially affect dendritic complexity and spine formation, suggesting a role for APOE genotypes not only in acute and chronic brain injuries including AD, but also in normal brain functions.

Results:

ApoE4 decreases while apoE3 increases spine density in primary hippocampal neurons

ApoE is involved in synapse and spine formation in primary cortical neurons (92). To measure the effects of apoE isoforms, we transfected primary hippocampal neurons with GFP, treated them with purified apoE3, apoE4, or PBS for 48 hours, and then analyzed spine density. We found that apoE4 decreased spine density (31±17.1%) and apoE3 increased spine density (82±18.3%) compared to the control (Fig.III.1A,B), consistent with another study (92). We further measured the length of spines from the base of the neck to the furthest point on the spine head (86). The cumulative distribution of spine length showed a significant shift in spine distribution towards shorter spines for apoE4 treated neurons compared to control and apoE3 treatment (Fig.III.1C). These findings suggest that apoE may play a critical role not only in determining the appropriate number of spines, but also in spine morphogenesis, and thus synaptic plasticity.
Fig.III.1. ApoE4 decreases spine density in primary hippocampal culture. A, Cultured hippocampal neurons (DIV 12) were transfected with GFP and treated with control (n=15), 500 nM apoE3 (n=15), or 500 nM apoE4 (n=15) for 48 hrs. Morphologies of dendritic spines were visualized by GFP fluorescence. Magnified examples of representative neuritic segments are shown in lower panels. B, Averaged spine density from data in A. C, The cumulative percentage distribution of dendritic spine length. ApoE4 treatment significantly shifted the distribution towards decreased spine length compared to control.
ApoE4 decreases spine density in vivo by 4 weeks of age

Previous studies have uncovered an association between the apoE4 genotype and spine deficits in vivo (35, 93-94). However, these studies did not compare all three apoE isoforms or examine their effects on dendritic spines in the cortex. To examine this, we initially focused on 4-week-old APOE TR mice, analyzing pyramidal neurons in cortical layers II/III using the rapid Golgi impregnation method. Because spine density may vary within both the dendritic field of a single neuron and between different neurons (95), the spines of the pyramidal neurons were counted from apical oblique (AO) and basal shaft (BS) dendrites (Fig. III.2A,B). The densities of AO, BS, and total (AO+BS) dendritic spines were significantly decreased in APOE4 TR mice by 24 to 30% compared to APOE3 and APOE2 TR mice (Fig. III.2C-E). APOE-deficient mice also had decreased AO, BS, and total spine densities by 28 to 33% compared to APOE3 and APOE2 TR mice (Fig. III.2C-E). These data show that APOE4 TR mice had reduced cortical dendritic spine density similar to mice lacking apoE at a young age.

Next, we examined the spine length to compare the morphology of individual dendritic spines in all three APOE TR mice and APOE-deficient mice. The cumulative distribution of spine length revealed a significant shift of APOE2 TR mice to longer spines compared to the other APOE TR and APOE-deficient mice (Fig. III.2F-H).
Fig. III.2. APOE4 decreases spine density in pyramidal cells in cortical layers II/III at 4 weeks of age. Mouse brains were Golgi stained and cortical layers II/III were imaged (n=4 mice/genotype). A, Representative Golgi impregnated neuron. AO: apical oblique and BS: basal shaft. B, Representative AO and BS dendrites per genotype C-E, Averaged spine densities for each genotype. C, Averaged total spine density, combining AO + BS dendrites (56 dendritic segments/group). D, Averaged spine density for AO spines (28 neurons/group). E, Averaged spine density for BS spines (28 neurons/group). F-H, The cumulative distribution percentage of spine length. APOE2 TR mice had a significant shift in distribution to longer spines compared to other groups (p<0.05, Kolmogorov-Smirnov test).
To test whether apoE isoforms differentially affected spine formation in the hippocampus, we analyzed spine density and morphology in granule cells of the dentate gyrus of the hippocampus (Fig. III.3A,B). The three APOE TR and the APOE-deficient mice did not alter the spine density in the dentate gyrus (Fig. III.3C), nor were there significant morphological shifts in the distribution of spine length (Fig. III.3D). We also examined the pyramidal cells in the CA1 region of the hippocampus (Fig 3E-G). Again, all three APOE TR and APOE-deficient mice showed similar spine densities for CA1 neurons, suggesting that the impact of APOE on spine formation may vary across brain regions.
Fig III.3. APOE isoforms exhibit similar spine density in the hippocampus at 4 weeks of age. Mouse brains were Golgi stained and granule cells in the dentate gyrus and CA1 neurons were imaged (n=4 mice/genotype). 

A, Left panel shows a Golgi impregnation of the hippocampus of an APOE3 TR mouse at 2.5x. The right panel is a representative image of APOE3 TR mice granule cells in the dentate gyrus at 10x magnification. 

B, Representative dendrites for the dentate gyrus per genotype. 

C, Averaged total spine density in the dentate gyrus (28 neurons/genotype). 

D, The cumulative percentage distribution plots of spine length. 

E, Representative image of APOE3 TR CA1 neurons at 10x magnification. 

F, Averaged data include quantification of AO (28 neurons/genotype) and G, BS (28 neurons/ genotype) spine number per 10 micrometer area.
**ApoE4 further reduces spine density in the cortex at 1 year**

To examine whether the effect of apoE isoforms on spine formation was age-dependent, we measured the spine density from all three APOE TR mice at 3 months and 1 year. Consistent with our observations at 4 weeks, APOE4 TR mice had decreased spine density at 3 months and a trend towards an even greater decrease at 1 year compared to APOE3 TR mice (Fig. III.4A,E, 24.4±8.8% and 55.6±10.5%, respectively). APOE2 TR mice showed similar spine densities to APOE3 TR mice at both ages (Fig. III.4A-C, E-G). For the spine morphology, we found that there are no significant differences in spine length distribution across groups at 3 months (Fig. III.4D). However, APOE4 TR mice had significantly shorter spines compared to the other APOE TR mice at 1 year of age (Fig. III.4H), consistent with our *in vitro* findings (Fig. III.1). Overall, these data suggest that the effect of apoE4 on spine formation may increase with aging in the cortex.
Fig. III.4. ApoE4 further reduces spine density in the cortex at 1 year. 

A, Averaged total spine density (56 neurons/group). 

B, Averaged AO spine density (28 neurons/group). 

C, Averaged BS spine density (28 neurons/group). *p<0.05 in comparison to APOE3.

D, Cumulative distribution of spine length. 

E-G, Mouse brains were Golgi stained and cortical layers II/III imaged at 1 year of age (n=4 mice/genotype). 

E, Averaged data include total spine density (56 neurons/genotype). 

F, Averaged data include AO spine density (28 neurons/genotype). 

G, Averaged data include BS spine density (28 neurons/genotype). * p<0.05 in comparison to APOE3.

H, Cumulative percentage distribution of spine length. APOE4 TR mice had a significant shift in distribution towards smaller spines compared to the other APOE TR (E3, E2) mice at 1 year of age.
We also tested whether apoE4 affected the spine density and morphology in the hippocampus with aging. We observed that all three APOE TR mice had similar spine densities at 3 months and 1 year for the dentate gyrus (Fig. III.5A,B). We also found that there was no significant shift in distribution for spine length from all three APOE TR mice at 3 months (Fig. III.5C). However, APOE2 TR mice had a significant shift in distribution to shorter spines in the dentate gyrus compared to APOE3 and APOE4 TR mice at 1 year (Fig.III.5D).
Fig III.5: APOE isoforms exhibit similar spine density in the hippocampus at 3 months and 1 yr of age. **A-B**, Mouse brains were Golgi stained and granule cells in the dentate gyrus were imaged at 3 months (n=4/genotype). **A**, Averaged total spine density for all genotypes (28 neurons/group). **B**, Cumulative percentage distribution of spine length. **C-D** Mouse brains were Golgi stained and granule cells in the dentate gyrus were imaged at 1 year (n=4). **C**, Averaged total spine density (28 neurons/group). **D**, Cumulative percentage distribution for spine length. APOE2 TR had a significant shift in distribution towards shorter spines compared to APOE (E3, E4) TR mice.
In summary, a comparison in cortical spine density for all three APOE TR mice over time (Fig III.6A) shows that by 4 weeks of age, APOE4 TR mice have a $27.7\pm7.4\%$ decrease in spine density compared to APOE3 TR mice. By 1 year, ApoE4 TR mice had a further decrease in spine density by $55.6\pm10.5\%$ compared to APOE3 TR mice (Fig. III.6A). This suggests an increasing trend of APOE4 effects on pyramidal spines in cortical layer II/III with aging. However, all three APOE TR mice had similar spine densities over time in the dentate gyrus (Fig. III.6B).
Fig. III.6. The effect of APOE4 on spine density demonstrated a trend towards increasing over time in cortical layers II/III. A, Summary of all the averaged spine density for all three APOE TR mice in cortical layer II/III at 4 weeks, 3 months, and 1 year. Spine density in APOE4 TR mice is decreased compared to APOE3 TR mice at 4 weeks (27.7 ± 7.4%), 3 months (24.4 ± 8.6%) and at 1 year (55.6 ± 10.5). B, Summary of the averaged spine density in APOE2, APOE3, and APOE4 TR mice in the dentate gyrus for all timepoints.
**ApoE4 decreases dendritic arborization in cortical neurons at 4 weeks**

ApoE3 increases dendritic arborization, while apoE4 decreases dendritic arborization *in vitro* (77). We tested whether similar results were found *in vivo*. We employed the Neurolucida software to trace dendrites of Golgi-stained pyramidal neurons in layers II/III of the somatosensory cortex at 4 weeks of age (Fig. III.7A). Quantitative analysis revealed that apoE isoforms significantly affected elaboration of apical dendrites but not basal dendrites of these neurons. Apical branching was significantly increased in APOE2 TR mice compared to APOE3 (25.5±14.5%) and APOE4 (39.8±11.7%) TR mice (Fig. III.7B). Furthermore, total apical dendritic length was significantly decreased in APOE4 compared to APOE2 TR mice (Fig. III.7C). Scholl analysis did not reveal a difference in the complexity of apical dendrites between APOE3 and APOE4 TR mice. However, we observed that APOE3 TR mice had significantly decreased apical branching at proximal distances from the soma, whereas APOE4 TR mice had significantly decreased apical branching distally from the soma (Fig. III.7D).
**Fig. III.7. ApoE4 decreases dendritic arborization in cortical neurons at 4 weeks.**

_A_, Three-dimensional graphical tracing representing dendrite morphology. The number of apical and basal dendrites was measured in micrometers, and the number of apical and basal nodes was counted. **B**, Averaged data include the node (branch) number for apical and basal dendrites for the 3 genotypes, analyzing 18 neurons per group. APOE2 mice had increased branching compared to APOE3 (25.5 ± 14.5%) and APOE4 (39.8 ± 11.7%). **C**, Averaged total dendritic length for apical and basal dendrites for the 3 genotypes, analyzing 18 neurons per group. APOE2 mice had significantly increased length (29.6 ± 9.6) compared to APOE4 TR mice. *p<0.05, **p<0.01** **D**, Using Sholl analysis, we graphed the average intersections per shell per neuron against the distance from the soma (micrometers). a = p < 0.05 for APOE3 vs. APOE2. b = p < 0.05 for APOE4 vs. APOE2.
Discussion

In the present study, we defined a physiological effect of apoE isoforms on spine formation \textit{in vivo}. We demonstrate that APOE-\(\varepsilon\)4 TR mice have reduced spine density and dendritic arborization in layer II/III of the cortex compared to APOE-\(\varepsilon\)2 and APOE-\(\varepsilon\)3 TR mice. We observed this deficit in three independent cohorts of mice at different ages (4 weeks, 3 months, and 1 year). These data suggest that apoE isoforms differentially affect spine formation and maintenance.

The effect of apoE isoforms on spine formation \textit{in vitro and in vivo}

Several studies have suggested that apoE may play an important role in promoting synapse formation and synaptic plasticity (77, 82). Indeed, we and others have found that apoE4 decreased spine density compared to apoE3 in primary neurons (Fig III.1) (92). Additionally, we and others have examined the effect of apoE4 on spine formation \textit{in vivo} (35, 93). We found that APOE-\(\varepsilon\)4 TR mice have decreased spine density compared to APOE-\(\varepsilon\)2 and APOE-\(\varepsilon\)3 TR mice in the cortex at three different ages (Fig. III.2 & 4). APOE-\(\varepsilon\)4 TR mice also have significantly reduced spine density compared to APOE-\(\varepsilon\)3 TR mice in the amygdala at seven months of age (35). Transgenic hAPP mice crossed with hAPOE-\(\varepsilon\)2 overexpressing mice exhibit increased dendritic spine density in CA1 neurons (94). Furthermore, APOE-\(\varepsilon\)4 transgenic mice (under control of the GFAP promoter) have reduced spine density at 12 months and 24 months but not at 3 weeks compared to APOE-\(\varepsilon\)3 transgenic mice in the dentate gyrus (93). We found no difference in spine density across all three APOE genotypes in the dentate gyrus, at all ages, or in
the CA1 region at 4 weeks (Fig. III.3 & 5). This discrepancy may be partially due to varying apoE levels found in the animal models used. For instance, we used APOE-ε4 TR mice, which exhibit reduced brain apoE levels compared to APOE-ε2 TR and APOE-ε3 TR mice (50), while Ji et al. used APOE-ε3 and APOE-ε4 mice (GFAP promoter), which express equal levels of apoE (93). Additionally, several studies have found that although apoE mRNA levels in the hippocampus and cortex are equivalent, total apoE protein levels are higher in the hippocampus compared to cortex regardless of APOE genotype for the APOE TR mice (50-51). The decreased baseline apoE levels in the cortex compared to the hippocampus for APOE-ε4 TR mice may account for the differential effects we observe in these brain regions. It is also possible that there is a discrepancy in expression levels of apoE receptors, which are implicated in synaptic plasticity, in the hippocampus vs. the cortex (Qiu, et al., 2006). Finally, we cannot completely exclude the possibility that the discrepancy may be due to a technical issue -- neurons in hippocampus are more densely packed compared to cortex; therefore imaged dendrites must be more distal from the soma compared to those imaged in cortex.

We are the first to analyze spine morphology by examining spine length across all APOE genotypes in vivo. We found a variation in cortical layers II/III across genotypes at three different time-points. Interestingly, APOE-ε2 TR mice had a significant distribution shift towards longer spines in the cortex at 4 weeks of age compared to the other APOE TR mice. This difference was not evident at later time-points, suggesting a role for apoE2 in the early development of spines. Conversely, APOE-ε4 TR mice had a significant distribution shift towards shorter spines compared to APOE-ε3 and APOE-ε2 TR mice at 1 year. This was not
observed at earlier time-points, suggesting that apoE4’s detrimental effects on spine morphology and spine density in the cortex may build over time.

How do apoE isoforms differentially regulate spine density? ApoE binds to a family of low density lipoprotein receptors (LDLRs), which are involved in neurite outgrowth and LTP (96). A recent study found differences in intracellular signaling cascades in all three APOE TR mice, suggesting that this may result from their differential binding affinities to this family of receptors (97). Another explanation could be that the apoE4 is deficient in providing neurons with high density lipid particles, which are necessary for dendritic spine remodeling and the formation of mature synapses (98).

The effect of apoE isoforms on neurite development

In previous studies, apoE4 decreased dendritic length and arborization compared to apoE3 in vitro (77, 82). However, we did not observe a significant difference in dendritic complexity between APOE3 and APOE4 TR mice, although some difference was noted. This may be due to several factors such as age and brain region examined. For example, we compared neurite arborization in APOE TR mice in the somatosensory cortical layer II/III at 4 weeks compared to the amygdala at 7 months in a previous study (35). Interestingly, we observed that APOE-ε3 and APOE-ε4 TR mice have decreased apical branching compared to APOE-ε2 TR mice (Fig. III.4). Moreover, APOE-ε4 TR mice have decreased apical dendritic length compared to APOE-ε2 TR mice (Fig. III.4). However, we did not observe a significant difference in basal dendrites across all genotypes. Similarly, there were alterations in apical but not basal dendritic
morphology compared to controls in a transgenic AD mouse model (95). This could be related to the fact that basal dendrites have less complexity compared to apical dendrites (99).

**Conclusion**

In conclusion, this study is the first to demonstrate that apoE isoforms have a differential regulatory effect on dendritic complexity and spine formation in cortical neurons. APOE-ε4 TR mice have reduced apical arborization compared to APOE-ε2 TR mice. Additionally, APOE-ε4 TR mice have reduced spine density compared to APOE-ε2 and APOE-ε3 TR mice. This reduction in spine density for APOE-ε4 TR mice increases over time, with significantly smaller spines compared to the other APOE TR mice at 1 year. These data may inform both normal brain functions of apoE as well as differential reparative responses, dependent on apoE isoform, to acute and chronic brain injuries, including AD.
Chapter IV: ApoE Receptor 2 Regulates Dendritic Spine Formation.\textsuperscript{b}

In the previous chapter, I have characterized how APOE genotype can differentially impact dendritic spine density. A potential mechanisms by which this may occur is through APOE receptor signaling. One of the APOE receptors, Apolipoprotein E receptor 2 (ApoEr2), is a postsynaptic receptor implicated in learning, and memory through unclear mechanisms (56). ApoEr2 can interact with synaptic cytoplasmic adaptor proteins such as PSD-95 (56-59), a major postsynaptic density protein important for synapse formation and function (60), and X11\(\alpha\), which is also involved in synapse formation (65-66).

Taken together, the data suggest that ApoEr2 may be intimately involved in synaptic regulation, implying that its ligand, ApoE, may also be involved in synaptic function. Therefore, ApoEr2 is an ideal receptor to begin examining whether it is a likely candidate for promoting dendritic spine formation. Here, we show that in primary neuronal cultures, overexpression of ApoEr2 increased dendritic spine density. In contrast, ApoEr2 knockout mice had decreased dendritic spine density in cortical layers II/III at 1 month of age, consistent with our \textit{in vitro} findings. We also tested whether the interaction between ApoEr2 and its cytoplasmic adaptor proteins, specifically X11\(\alpha\)/mint-1 and PSD-95, affected synapse and dendritic spine formation. X11\(\alpha\) decreased cell surface levels of ApoEr2 along with synapse and dendritic spine density. In contrast, PSD-95 increased cell surface levels of ApoEr2 as well as synapse and dendritic spine density.

\textsuperscript{b} This chapter has been modified from Dumanis SB, Cha H-J, Song JM, Trotter JH, Spitzer M, Lee JY, Weeber EJ, Turner RS, Pak DTS, Rebeck GW, Hoe HS (2011) ApoE Receptor 2 Regulates Synapse and Dendritic Spine Formation. PLoS ONE 6(2): e17203(2): e17203
Results

ApoEr2 expression in synapses

ApoEr2 is expressed in neurons throughout the mammalian brain (100). To determine whether ApoEr2 is expressed at the synapse, primary hippocampal neurons were immunostained with anti-ApoEr2 and anti-PSD-95 antibodies (Fig. IV.1A). ApoEr2 immunoreactivity was highest in pyramidal neurons, where it was found to have a punctate distribution throughout dendritic processes, and where it co-localized with the postsynaptic marker PSD-95, suggesting that ApoEr2 is present in synapses (Fig. IV.1A).

We also conducted synaptosomal fractionation on adult mouse forebrains. To monitor the purity of synaptosomal fractionations, we used PSD-95 as a postsynaptic marker and synaptophysin as a presynaptic marker. We then examined whether X11α and PSD-95, which interact with ApoEr2 (61-62), are present in the pre- or postsynaptic fractions. We found that X11α was present in both pre- and postsynaptic fractions, while ApoEr2 and PSD-95 specifically localized to postsynaptic density fractions (Fig. IV.1B).

We next examined the expression levels of ApoEr2 and its cytoplasmic adaptor proteins during CNS development in mice. The levels of ApoEr2 in the brain increased markedly between embryonic day 17 and postnatal day 10 (Fig. IV.1C). X11α was detectable beginning at postnatal day 10 and increased until postnatal day 36; this period (postnatal day 10-36) is known to be important for synapse maturation (Fig. IV.1C). Levels of PSD-95 further increased between postnatal days 1 and 36, an important period for synaptogenesis, compared to embryonic stages (Fig. IV.1C). These data demonstrate age-dependent increases in levels of ApoEr2, PSD-95, and...
X11α, and reveal overlapping, but nonidentical, expression profiles among these proteins. The early peak in ApoEr2 levels suggests that it plays important roles in neuronal development, while sustained expression of ApoEr2 suggests its potentially ongoing involvement in adult plasticity.
Figure IV.1. ApoEr2 is expressed at synapses. A. Primary hippocampal neurons at DIV 14 were immunostained for ApoEr2 and PSD-95. Primary antibodies were detected with Alexa Fluor 488 anti-rabbit (in green; middle panel) and Alexa Fluor 594 anti-mouse (in red; left panel). Immunolabeled neurons were imaged by confocal microscopy (63X). Colocalization of ApoEr2 and PSD-95 appears as yellow in the right panel. White bar represents 10 micrometers. B. We performed immunoblot analysis on ApoEr2, X11α, PSD-95, and synaptophysin in presynaptic vesicles (SV) and postsynaptic density (PSD) fractions. X11α is present in both pre- and postsynaptic fractions; synaptophysin is present in presynaptic fractions and PSD-95 is present in post-synaptic fractions. ApoEr2 is predominantly found in the postsynaptic fraction. C. Levels of ApoEr2, X11α, PSD-95, and β-actin (used as protein loading control), were examined by immunoblot at developmental stages (n=3 per timepoint). ApoEr2 levels increased at E17-P6, a critical period for synaptogenesis.
**ApoEr2 promotes dendritic spines in primary hippocampal neurons**

To examine the postsynaptic effects of ApoEr2, specifically, the effects of ApoEr2 on dendritic spine formation, primary hippocampal neurons (transfected at DIV 14, expressed for 3 days) were transfected with GFP and empty vector or GFP and ApoEr2, and spine density was measured. We found that overexpression of ApoEr2 significantly increased spine number (20 ± 3% increase) compared to GFP alone (n=15) (Fig. IV.2 C, D), suggesting that ApoEr2 plays a role in dendritic spine formation.
Figure IV.2. ApoEr2 promotes dendritic spine formation in primary hippocampal neurons. A. Cultured hippocampal neurons were transfected with GFP and empty vector or GFP and ApoEr2-HA as indicated. Morphology of neurons and dendritic spines were visualized by GFP fluorescence. Magnified examples of representative dendritic segments are shown in bottom panels. B. Quantification of spine density from A, with asterisks defining statistically significant differences from GFP-transfected cells (n=15, *p<0.05). Error bars are represented as S.E.M. White bar represents 10 micrometers.
To further examine whether ApoEr2 regulates dendritic complexity, primary hippocampal neurons (DIV 7) were transfected with GFP-β-actin and empty vector or GFP-β-actin and ApoEr2-HA. We immunostained with GFP and then conducted DAB staining to examine neuronal morphology. Using sholl analysis, we measured dendritic complexity at incremental lengths from the soma. We found that overexpression of ApoEr2 did not increase dendritic complexity compared to controls (Fig. IV.3). Additionally, overexpression of ApoEr2 did not significantly affect total dendritic length or number compared to controls (data not shown). These data suggest that ApoEr2 does not alter dendritic complexity; however, it may regulate dendritic spine formation.
Figure IV.3 ApoEr2 does not alter dendritic complexity compared to controls. Primary hippocampal neurons (DIV 7) were transfected with GFP-β-actin and empty vector or GFP-β-actin and ApoEr2-HA. On DIV 14, neurons were immunostained with GFP followed by DAB to visualize cell morphology. A. Three-dimensional graphical tracing representing dendrite morphology for control (left panel) and ApoEr2 (right panel). Line bar represents 50 µm length. B. Using Sholl analysis of cells in (A), we graphed the average intersection per shell per neuron against the distance from the soma (in micrometers). (n=10 neurons/condition)
The extracellular and intracellular domains of ApoEr2 are important for dendritic spine formation

Our previous findings demonstrated that ApoEr2 promotes dendritic spine formation (Fig. IV. 2 C, D). Therefore, we then examined which domains of ApoEr2 are essential to this process. To test this, we used deletion constructs of ApoEr2, as previously described (Fig IV.4A) (101-102). Primary hippocampal neurons were transfected with GFP and empty vector (#1), GFP and ApoEr2 lacking the ligand binding domain (#2), GFP and ApoEr2 lacking the ligand binding and EGF binding domains (#3), GFP and ApoEr2 lacking the ligand binding, EGF binding and O-linked domains (#4), and GFP and full-length ApoEr2 (#5), and spine density was measured (Fig IV.4B). Consistent with our previous findings, full-length ApoEr2 (#5) significantly increased dendritic spine density by 36% compared to GFP transfected cells (#1) (Fig. IV.2C, D, 4B, C). However, none of the constructs lacking the ligand binding domain of ApoEr2 increased dendritic spine formation (Fig. IV.4B, C). We further examined the specificity of the ligand binding domain of ApoEr2 in dendritic spine formation, we used a second set of deletion constructs of ApoEr2 with a C-terminal HA tag (Fig IV.4D). For these experiments, primary hippocampal neurons were transfected with GFP and empty vector (#1), GFP and a secreted form of ApoEr2 lacking the transmembrane and intracellular domains (#7), and GFP and full-length ApoEr2 (#6). Consistent with previous results, full-length ApoEr2 (#6) significantly increased dendritic spine density compared to GFP. Interestingly, the extracellular domain of ApoEr2 (#7) did not significantly increase dendritic spine density compare to controls, suggesting that the ligand binding domain of ApoEr2 alone is not sufficient to induce spine formation (Fig. IV.4 E,F).
Figure IV.4. Extracellular and intracellular domains of ApoEr2 are essential for dendritic spine formation. A. Deletion constructs of ApoEr2 with a c-terminal myc tag. B. Primary hippocampal neurons (DIV 14) were transfected with GFP and empty vector (#1), GFP and ApoEr2 lacking a ligand binding domain (#2), GFP and ApoEr2 lacking ligand binding and EGF binding domains (#3), GFP and ApoEr2 lacking ligand binding, EGF binding and O-linked domains (#4) and GFP and full-length ApoEr2 (#5). After 48 hours, neurons were fixed and stained for GFP. Morphology of neurons and dendritic spines was visualized by GFP fluorescence. Magnified examples of representative dendritic segments are shown in bottom panels. C. Quantification of data shown in B (n=15/per groups, ***, p<0.001). Error bars are represented as S.E.M. White bar represents 10 micrometers. D. Deletion constructs of ApoEr2 with a c-terminal HA tag. E. Primary hippocampal neurons (DIV 14) were transfected with GFP and empty vector (#1), GFP and full-length ApoEr2 (#6), or GFP and extracellular domain of ApoEr2 (#7). After 48 hours, neurons were fixed and stained for GFP. Morphology of neurons and dendritic spines was visualized by GFP fluorescence. Magnified examples of representative dendritic segments are shown in bottom panels. F. Quantification of data shown in C (n= 10/per group, **, p<0.005). Error bars are represented as S.E.M. White bar represents 10 micrometers.
**ApoEr2 deficient mice have decreased spine density at 1 month of age**

To determine whether ApoEr2 has a similar effect on dendritic spine formation *in vivo*, we analyzed pyramidal neurons in cortical layers II/III using the rapid Golgi impregnation method in ApoEr2 knockout mice and wild-type littermates at 1 month old. Because spine density may vary within both the dendritic field of a single neuron and between different neurons (103), the spines of the pyramidal neurons were counted as apical oblique (AO), basal shaft (BS), and total (AO+BS) dendrites (Fig. IV.5A, B). We found that ApoEr2 knockout mice had significantly reduced dendritic spine number on AO, BS, and total dendrites compared with wild-type littermates at 1 month old (Fig. IV.5C–E).

Next, we examined the morphology of dendritic spines by measuring spine width and length in ApoEr2 knockout mice and wild-type littermates at 1 month old. The cumulative distributions revealed no significant differences in spine width between ApoEr2 knockout mice and wild-type mice (Fig IV.5F-H). However, we observed a significant shift in 1 month old ApoEr2 knockout mice to shorter spines compared to wild-type mice in the AO, but not BS dendrites (IV.5I-K, p<0.05).
Figure IV.5. ApoEr2 deficient mice have reduced dendrite spine density in pyramidal neurons in cortical layers II/III at 1 month of age. Mouse brains were Golgi stained and cortical layers II/III imaged (n = 4 mice/genotype). A. Representative Golgi-impregnated pyramidal neurons in cortical layer II/III of a wild-type (left panel) and ApoEr2 knockout mice (right panel). B. Representative AO and BS dendrites per genotype. Black bar indicates a 20 micrometer scale. C–E. Averaged spine densities for each genotype. C. Averaged spine density for AO spines (28 neurons/group). D. Averaged spine density for BS spines (28 neurons/group). E. Averaged total spine density, combining AO + BS dendrites (56 dendritic segments/group). Error bars are represented as S.E.M. *, p < 0.05. F-H. The cumulative distribution percentage of spine width. There were no significant shifts in distribution between ApoEr2 knockout and wild-type littermates. I–K. The cumulative distribution percentage of spine length. ApoEr2 knockout mice had a significant shift to smaller spines compared to wild-type mice in the AO (I) and Total (K) but not BS (J). (p < 0.05, Kolmogorov–Smirnov test).
We further examined whether the effects of ApoEr2 on dendritic spine density was age-dependent. To test this, we conducted Golgi staining on ApoEr2 knockout mice and wild-type littermates at 1 year of age (Fig IV.6A, B). Interestingly, we observed no significant differences in dendritic spine densities between ApoEr2 knockout mice and wild-type littermates in cortical layers II/III at 1 year old (Fig IV.6C-E). This data suggests the existence of compensatory mechanisms between one month of age and one year of age in ApoEr2 knockout mice. We also conducted morphological analysis in 1 year old ApoEr2 knockout mice and wild-type littermates. The cumulative distributions revealed a significant shift in ApoEr2 knockout mice to shorter spines compared to wild-type mice in the AO, but not BS dendrites (data not shown, p<0.05 for AO condition), consistent with our 1 month of age studies.
Figure IV.6. ApoEr2 knockout mice have no change in spine density in pyramidal neurons in cortical layers II/III at 1 year of age. Mouse brains were Golgi stained and neurons in cortical layers II/III imaged (n = 4 mice/genotype). A. Representative Golgi-impregnated pyramidal neuron in cortical layer II/III of a wild-type (left panel) and ApoEr2 knockout mice (right panel). B. Representative AO and BS dendrites per genotype. Black bar indicates 20 micrometer scale. C–E. Averaged spine densities for each genotype. C. Averaged total spine density, combining AO + BS dendrites (56 dendritic segments/group). D. Averaged spine density for AO spines (28 neurons/group). E. Averaged spine density for BS spines (28 neurons/group). Error bars are represented as S.E.M.
**ApoEr2 increased clustering of synaptic proteins**

Because our data indicate that ApoEr2 plays an important role in promoting dendritic spine numbers, we examined whether ApoEr2 affects clustering of synaptic proteins at the post synaptic terminal. For these experiments, primary hippocampal neurons were infected with ApoEr2 sindbis virus or GFP for 20 hours and intensity or puncta number of PSD-95 and synaptophysin were measured. We found that ApoEr2 significantly increased PSD-95 intensity throughout the cultures by 38% compared to GFP infected neurons (Fig IV.7A, B). We also found that, although ApoEr2 did not alter total synaptophysin levels, it did significantly increase puncta number of synaptophysin along the neuronal processes by 78% compared to control (Fig IV.7C, D). These data suggest that, while ApoEr2 does not immediately alter the level of synaptophysin, it does affect the localization of synaptophysin.

Because AMPA receptors are important for synaptic plasticity and dendritic spine formation, we examined whether ApoEr2 affected total levels of AMPA receptor subunits by infecting neurons with ApoEr2 or GFP (104-106). We found that ApoEr2 infected neurons had significantly decreased total GluA1 intensity by 25% along the neuronal process (Fig IV.7E,F) and significantly increased total GluA2 intensity by 66% compared to GFP infected neurons (Fig IV.7G,H). These data suggest that ApoEr2 differentially regulates AMPA receptor subunit expression levels.
Figure IV.7. ApoEr2 regulates synaptic protein levels. A. Cultured hippocampal neurons (DIV 14) were infected with ApoEr2 or GFP sindbis virus for 20 hours and stained for PSD-95. B. Quantification of average puncta intensity in (A) (10 neurons/group, *** $p < 0.001$). C. Cultured hippocampal neurons (DIV 14) were infected with ApoEr2 or GFP sindbis virus for 20 hours, and stained for synaptophysin. D. Quantification of average intensity (left) and puncta number (right) in (C) (10 neurons/group, ** $p < 0.005$). E. Cultured hippocampal neurons (DIV 14) were infected with ApoEr2 or GFP sindbis virus for 20 hours and stained for GluA1. F. Quantification of average intensity in (E) (10 neurons/group, ** $p < 0.005$). G. Cultured hippocampal neurons (DIV 14) were infected with ApoEr2 or GFP sindbis virus for 20 hours and stained for GluA2. H. Quantification of average intensity in (G) (10 neurons/group, ** $p < 0.005$). Error bars are represented as S.E.M. White bar represents 10 micrometers.
ApoEr2 increases surface levels of GluA2 and decreases cell surface levels of GluA1

We then further examined whether ApoEr2 can regulate the distribution of AMPA receptor subunits on the cell surface. To test this, primary hippocampal neurons were transfected with GFP and empty vector or GFP and ApoEr2 and live cell surface staining was conducted. We found that ApoEr2 significantly decreased cell surface GluA1 by 65% (Fig IV.8A, B), but significantly increased cell surface of GluA2 by 43% (Fig IV.8 C, D). These data further suggest that ApoEr2 differentially regulates AMPA receptor subunit distribution.
Figure IV.8. *ApoEr2 decreases cell surface GluA1 and increases surface level of GluA2.* A. Cultured hippocampal neurons (DIV 14) were transfected with GFP and empty vector or GFP and ApoEr2-myc for 48 hours and stained with an antibody recognizing the N terminus of GluA1 under impermeable conditions. B. Quantification of cell surface GluA1 levels in (A) (n=15 neurons/group, *p <0.05). C. Cultured hippocampal neurons (DIV 14) were transfected with GFP and empty vector or GFP and ApoEr2-myc for 48 hours and stained with an antibody recognizing the N terminus of GluA2 under impermeable conditions. D. Quantification of cell surface GluA2 levels in (C) (n=15 neurons/group, *p <0.05). Error bars are represented as S.E.M. White bar represents 10 micrometers.
**X11α decreases cell surface ApoEr2 and inhibits ApoEr2 induction of spine formation.**

We and others have demonstrated that ApoEr2 interacts with cytoplasmic adaptor proteins, specifically X11α and PSD-95 (58, 61, 107). Initially, we wanted to determine whether the interaction between ApoEr2 and X11α affected cell surface levels of ApoEr2. To test this, COS7 cells were transfected with ApoEr2 and empty vector or ApoEr2 and X11α for 24 hours. Cell surface proteins were biotinylated, isolated with avidin beads, and immunoblotted for ApoEr2. We found that co-transfection with X11α decreased cell surface levels of ApoEr2 in COS7 cells (Fig IV.9A). Additionally, we conducted live cell surface staining by overexpressing ApoEr2 and empty vector or ApoEr2 and X11α in primary hippocampal neurons. We found that X11α decreased cell surface levels of ApoEr2 by 54% in primary hippocampal neurons (Fig. IV.9B, C, P<0.05; n=13). Thus, two independent assays suggested that X11α can modulate the effects of ApoEr2 on cell surface expression.

Next, we examined whether X11α could regulate the effect of ApoEr2 on dendritic spine formation in primary hippocampal neurons. To test this, primary hippocampal neurons (DIV 14) were transfected with GFP and empty vector, GFP with ApoEr2-HA and empty vector, GFP with ApoEr2-HA and X11α, or GFP with X11α and empty vector for 48 hours. After 48 hours, we conducted immunostaining with GFP (for morphological analysis). ApoEr2 significantly increased dendritic spine number (Fig.IV.9F and G, by 21%, n=15, p<0.05) compared to GFP alone consistent with our earlier findings (Fig. IV.2). In addition, we found that co-transfection with ApoEr2 and X11α significantly reduced dendritic spine density (32% decrease, n=15, p<0.05) compared to ApoEr2 alone (Fig. IV. 9F and G).
Figure IV.9. X11α decreases cell surface ApoEr2 and inhibits ApoEr2-induced synapse and dendritic spine formation. A. COS7 cells were transfected with GFP-ApoEr2 and vector (lane 1, 3) or X11α (lane 2, 4). Cell surface proteins were biotin-labeled, isolated with avidin-beads, and immunoblotted with GFP for ApoEr2. Full length X11α decreased surface levels of ApoEr2 (upper blot). Cell lysates showed similar levels of total ApoEr2 (lower blot). B. Cultured hippocampal neurons were transfected at DIV12 with either GFP-ApoEr2 or empty vector (upper panel) or GFP-ApoEr2 and X11α (lower panel). Surface ApoEr2 was measured with GFP at DIV14 by immunofluorescence of live cells. Left panels, GFP-ApoEr2; right panels, surface ApoEr2. C. Quantification of B. D. Cultured hippocampal neurons (DIV 14) were transfected with GFP and empty vector, GFP and ApoEr2-HA and empty vector, GFP and ApoEr2-HA and X11α, or GFP and X11α and empty vector as indicated. After 48 hours, morphology of neurons and dendritic spines were visualized by GFP fluorescence. Magnified examples of representative dendritic segments are shown in lower panels. E. Quantification of spine density from (D), with asterisks defining statistically significant differences from GFP-transfected cells (n=10, p <0.05). Error bars are represented as S.E.M. White bar represents 10 micrometers.
**PSD-95 increases cell surface ApoEr2 and enhances ApoEr2 induction of spine formation.**

In our previous study, PSD-95 increased cell surface ApoEr2 levels in COS7 cells and in HEK293 cells (58). For the present study, we further examined whether PSD-95 could alter cell surface levels of ApoEr2 in primary hippocampal neurons. To test this, primary hippocampal neurons were transfected with GFP-ApoEr2 fusion protein and vector or GFP-ApoEr2 and PSD-95 and live cell surface staining was conducted (Fig IV.10A). We found that PSD-95 significantly increased cell surface levels of ApoEr2 by 49% in primary hippocampal neurons compared to controls (Fig IV.10B).

We further tested whether PSD-95 could regulate the effect of ApoEr2 on dendritic spine formation. To test this, primary hippocampal neurons (DIV 14) were transfected with GFP and empty vector, GFP and ApoEr2-HA and empty vector, GFP and ApoEr2-HA and PSD-95, or GFP and PSD-95 and empty vector. After 48 hours, we conducted immunostaining with anti-HA (to measure total levels of ApoEr2) and GFP (for morphological analysis). We found that ApoEr2 significantly increased dendritic spine density by 27% compared to GFP (Fig. IV.10 E, F). Additionally, PSD-95 increased dendritic spine density by 21% compared to GFP -- consistent with previous reports (108). Interestingly, co-transfection of PSD-95 with ApoEr2 further increased the number of dendritic spines compared to ApoEr2 alone (Fig. IV.10E, F). These results suggest that interactions between ApoEr2 and PSD-95 can regulate synapse and spine formation by modulating surface ApoEr2 levels.
Figure IV.10. PSD-95 increases cell surface ApoEr2 and increases ApoEr2-induced synapse and dendrite spine formation. A. Cultured hippocampal neurons were transfected at DIV12 with either GFP-ApoEr2 or empty vector (upper panel) or GFP-ApoEr2 and PSD-95 (lower panel). Surface ApoEr2 was measured with GFP at DIV14 by immunofluorescence of live cells. Left panels, GFP-ApoEr2; right panels, surface ApoEr2. B. Quantification of surface ApoEr2 intensity in neuronal processes in (A). PSD-95 increased cell surface levels of ApoEr2 by 49% (n=10, *p <0.05). C. Cultured hippocampal neurons were transfected at DIV 14 with GFP and empty vector, GFP and ApoEr2 and empty vector, GFP and ApoEr2 and PSD-95, or GFP and PSD-95 and empty vector as indicated. 48 hours later, morphology of neurons and dendritic spines were visualized by GFP fluorescence. Magnified examples of representative dendritic segments are shown in lower panels. D. Quantification of spine density from (C), with asterisks defining statistically significant differences from GFP-transfected cells (n=12, *p <0.05). Error bars are represented as S.E.M. White bar represents 10 micrometers.
To ensure that expression of total ApoEr2 was consistent across conditions, we repeated the experiment as described above. 48 hours later, we conducted immunostaining for anti-HA (to measure total levels of ApoEr2) and GFP (for morphological analysis). We observed no significant differences in total ApoEr2 levels across conditions (Fig. IV. 11). These data suggest that ApoEr2 induces spine formation, but this function is regulated by interaction with PSD-95 and X11α.
Figure IV.11 ApoEr2 total expression levels were consistent across conditions. Primary hippocampal neurons (DIV 14) were transfected with GFP and empty vector, GFP with ApoEr2-HA and empty vector, GFP with ApoEr2-HA and X11α, GFP with X11α and empty vector, or GFP with PSD95 and empty vector for 48 hours. HA staining was performed to measure total levels of ApoEr2 and GFP staining was performed for morphological analysis. A. Representative images for the conditions indicated. Primary antibodies were detected with Alexa Fluor 488 anti-rabbit for GFP (top panel) and Alexa Fluor 594 anti-mouse for HA (bottom panel). Immunolabeled neurons were imaged by confocal microscopy (63X). White bar represents 20 micrometers in length. B. Quantification of average HA intensity from (A). Error bars are represented as S.E.M.
Discussion

In the present study, we defined a physiological function of ApoEr2 at synapses. We demonstrated that ApoEr2 is expressed postsynaptically. Moreover, ApoEr2 significantly increases dendritic spine density in primary hippocampal neurons, suggesting that ApoEr2 also contributes to spine development. We also examined how interaction between ApoEr2 and the synaptic adaptor proteins X11α and PSD-95 modulated ApoEr2-induced effects on dendritic spine formation. We found that X11α decreased, while PSD-95 increased, cell surface ApoEr2 levels. Additionally, we found that X11α inhibited, while PSD-95 enhanced ApoEr2-induced effects on spines. These results demonstrate that ApoEr2 is important for dendritic spine formation, and that this effect can be further modulated via interaction with its cytoplasmic adaptor proteins.

ApoEr2 plays an important role in induction of LTP, learning and memory, and synaptic transmission in adult brain (56, 109). These processes require the cytoplasmic, alternatively spliced exon 19 of ApoEr2 (56). In this study, we demonstrated the ability of ApoEr2 to promote dendritic spine formation in primary hippocampal neurons. To examine the effects of ApoEr2 on dendritic spine formation in vivo, we conducted a Golgi rapid impregnation staining in ApoEr2 knockout mice and wild-type littermates at 1 month and 1 year of age. We found that 1 month old ApoEr2 knockout mice had significant deficits in spine number compared to controls (Fig. IV.5). However, 1 year old ApoEr2 knockout mice had similar spine densities compared to controls, suggesting that there may be compensatory mechanisms following initial spine formation in ApoEr2 knockout mice. One possible compensatory mechanism is modulation of
extracellular ligands for ApoEr2, such as apolipoprotein E (apoE) or Reelin, which are known to be important for dendritic spine formation (85, 110-112). Perhaps apoE and Reelin levels increased to compensate for ApoEr2 deficiency in ApoEr2 knockout mice, resulting in the similar spine densities compared to wild-type littermates that we observed at 1 year. It is also possible that ApoEr2 plays an important role in the initial formation of dendritic spines, but not their maintenance in adulthood. These possibilities can be further examined in the study of this receptor and its ligands as a function of age.

In the present study, we examined how ApoEr2 affects synapse formation by measuring synaptic protein levels of synaptophysin and PSD-95 in ApoEr2-infected neurons compared to controls. We observed that ApoEr2 infected neurons increased levels of PSD-95 and increased puncta of synaptophysin. Additionally, ApoEr2 overexpression increased protein levels of SPAR, a Rap-specific GAP that promotes spine formation (data not shown) (86, 113). These data suggest that ApoEr2 may alter the number of synaptic sites in cultured hippocampal neurons by regulating the assembly of a complex of postsynaptic proteins. We also examined the effects of ApoEr2 on total and cell surface levels of AMPA receptor subunits and observed that ApoEr2 caused an increase in total and cell-surface levels of GluA2, but a decrease in total and cell surface levels of GluA1. These results are somewhat surprising given that GluA1/A2 heteromers comprise the predominant AMPA receptor population in the hippocampus (114-115). Our findings suggest that ApoEr2 may alter the subunit composition of AMPA receptors from a GluA1/A2 to a GluA2/A3 combination. While neither GluA1 nor GluA2 is absolutely required for normal dendritic spine formation or morphogenesis in vivo (116-117), GluA2 overexpression has been shown to enhance dendritic spine formation in hippocampal cultured neurons (106).
Thus, the preferential increase in GluA2 by ApoEr2 could reflect a mechanism to stimulate spinogenesis during development.

Alternatively, a shift from GluA1 to GluA2-containing receptors could be related to differential cell biological properties of the two receptor subunits. For example, GluA1 has been suggested to require activity-dependent stimulation for acute delivery, whereas GluA2 may undergo constitutive trafficking and turnover (104, 118). In addition, GluA1 channel conductance or opening is enhanced by phosphorylation during various forms of synaptic plasticity such as LTP (119). Furthermore, the great majority of GluA2 exists in a calcium impermeable isoform, such that regulation of GluA2 levels can serve as a mechanism to limit calcium permeability of AMPA receptors (120). Taken together, these divergent properties suggest that decreased GluA1 may lead to a reduced ability to undergo activity-dependent synaptic potentiation, perhaps as a compensatory response to the increase in number of excitatory synapses and dendritic spines generated by elevated GluA2. A better understanding of the functional consequences of AMPA receptor subunit composition shifts observed here will require additional studies conducting detailed electrophysiological analysis of ApoEr2-expressing neurons.

The definitive mechanism by which ApoEr2 acts remains unknown. It is possible that ApoEr2 modulates dendritic spine morphology by promotion of de novo spine formation or by stabilization of existing dendritic spines. We hypothesize that ApoEr2 may regulate spine formation through interaction with Reelin (111) or ApoE, as well as through interaction with cytoplasmic adaptor proteins X11α and PSD-95. Supporting this hypothesis, we observed that the ligand binding domain of ApoEr2 is essential, but not sufficient for spine formation,
suggesting that extracellular interaction with Reelin or APOE may be part of the mechanism by which ApoEr2 increases dendritic spine number. Furthermore, we found that co-transfection with ApoEr2 and PSD-95 enhanced dendritic spine formation as compared to ApoEr2 alone, as opposed to co-transfection with ApoEr2 and X11α, which decreased spine formation as compared to ApoEr2 alone. However, further investigation is necessary to determine how ApoEr2 affects synapses and spine formation.

In conclusion, our results demonstrate, for the first time, that ApoEr2 promotes dendritic spine formation in vitro and in vivo, an effect further regulated by interaction with cytoplasmic adaptor proteins. These findings provide a better understanding of the physiological actions of ApoEr2 in the normal brain. We speculate that these molecular roles may be relevant to ApoEr2 functions in synaptic plasticity and learning and memory, both of which depend on the dynamic or persistent formation or elimination of synapses and dendritic spines.
Chapter V: APOE genotype affects the biochemistry at presynaptic compartments of glutamatergic nerve terminals

Using APOE knock-in mice, we have shown in chapter I that APOE-ε4 Targeted Replacement (TR) mice have fewer dendritic spines and reduced branching in cortical neurons. Since dendritic spines are postsynaptic sites of excitatory neurotransmission, we then used APOE TR mice to examine whether APOE genotype affected the various elements of the glutamate-glutamine cycle. We found that levels of glutamine synthetase and glutamate uptake transporters were unchanged among the APOE genotypes. However, compared to APOE-ε3 TR mice, APOE-ε4 TR mice had decreased glutaminase levels (18%, p<0.05), suggesting decreased conversion of glutamine to glutamate. APOE-ε4 TR mice also had increased levels of the vesicular glutamate transporter VGLUT1 (20%, p<0.05), suggesting that APOE genotype affects presynaptic terminal composition. To address whether these changes affected normal neurotransmission, we examined the production and metabolism of glutamate and glutamine at 4-5 months and 1 year. Using high frequency $^{13}$C / $^1$H nuclear magnetic resonance (NMR) spectroscopy, we found that APOE-ε4 TR mice have decreased production of glutamate and increased levels of glutamine. Taken together, these data suggest that APOE genotype affects presynaptic terminal composition and impacts the normal GLU-GLN cycle. These factors may contribute to the increased risk of neurodegeneration associated with APOE-ε4, and also act as surrogate markers for AD risk.

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c This chapter has been modified from Dumanis SB, DiBattista AM, Miessau M, Moussa CE, Rebeck GW (2013). APOE genotype affects the presynaptic compartment of glutamatergic nerve terminals. Journal of Neurochemistry. 124(1) : p4-14.
Results

Brain glutaminase levels are lower in APOE-ε4 TR mice

The APOE TR mice show differential effects on spine density and neuronal morphology, with APOE-ε4 TR mice having reduced dendritic complexity and spine density compared to APOE-ε3 and APOE-ε2 TR mice (35, 110). Dendritic spines are postsynaptic sites of excitatory neurotransmission, and glutamate (GLU) is the primary excitatory neurotransmitter in the brain. Glutamine (GLN) derived GLU from the GLU-GLN cycle is necessary for the maintenance of GLU at the nerve terminals (121-122). Therefore, we asked whether APOE genotype impacted the GLU-GLN cycle.

We examined the brains of APOE TR mice at 4-5 months of age for levels of glutamine synthetase, the enzyme responsible for converting GLU to GLN. Brains were extracted in buffers to isolate soluble and membrane bound proteins, and immunoblotted for glutamine synthetase. Glutamine synthetase was present in both the cytoplasm and membrane bound fractions (Fig V.1A, upper panel), but there were no significant differences in the levels between the APOE-ε3 and APOE-ε4 TR mouse brains in either fraction (Fig V.1A-C).

We also tested the levels of phosphate-activated glutaminase (PAG), the enzyme responsible for converting GLN to GLU. PAG was more prevalent in the membrane bound fraction of brain extracts (Fig V.1D, upper panel), consistent with its partial location at the outer face of the inner membrane wall of mitochondria (123). In brains of 4-5 month old mice, the membrane bound levels of PAG were significantly lower in the APOE-ε4 TR mice than the APOE-ε3 TR mice (23%, p<0.05) (Fig V.1E). We tested these results in a second cohort of 4-5
month old mice. Consistent with Fig V.1, we found that PAG was significantly lower in the membrane fraction of APOE-ε4 TR brains compared to APOE-ε3 TR brains (18%, p<0.05; data not shown). The cytoplasmic levels of PAG in APOE TR were not significantly different between the genotypes (Fig. V.1F).
Figure V.1: Glutaminase is reduced in APOE-ε4 TR mice. 4-5 month old APOE TR mouse brains (males) were homogenized by TBS and then TBS-X by sequential fractionation. Homogenates were probed for glutamine synthetase (A-C) and glutaminase (D-F). (A) A representative immunoblot showing that Glutamine Synthetase is present in both the TBS and TBSX fraction of these mice (upper panel). A representative blot showing the levels of Glutamine Synthetase in the TBSX Fraction (middle panel) and the TBS fraction (lower panel). Tubulin was used as a loading control. (B) Quantification of glutamine synthetase levels in the TBS-X and (C) TBS fractions. (D) Representative immunoblot showing that glutaminase is present in the TBSX and TBS fractions. (E) Quantification of the Glutaminase levels in the TBSX and (F) TBS fractions. n=5 per genotype. *, p<0.05 compared to APOE-ε3 TR mice. Error bars expressed as S.E.M.
To determine whether APOE-ε4 TR mice had differences in the brain distribution of PAG compared to APOE-ε3 TR mice, we performed an immunostain for PAG in coronal brain sections. PAG was observed predominantly in the soma of neuronal cells in layers II/III and in layer V of the cortex. There was also intense staining for glutaminase in the dentate gyrus, the stratum moleculare and the stratum oriens of the hippocampus consistent with previous reports (124) (Fig V.2). No differences in the distribution of PAG were observed between the APOE genotypes (Fig V.2).
Figure V.2: Glutaminase regional distribution is similar in the APOE-ε4 and APOE-ε3 TR mice. 6 month old male APOE TR mouse brain sections (50 microns thick) were stained and processed via peroxidase immunohistochemistry for glutaminase staining and cresyl violet counter-staining. (A-C) APOE3 TR representative image and (D-F) APOE4 TR representative image (A,D). Image at 2X magnification, scale bar to 500 microns. (B,E) Cortical image and (C,F) Hippocampal image of the dentate gyrus at 20x magnification. Scale bar set to 100 microns.
Brain VGLUT1 levels are significantly higher in APOE-ε4 TR mice.

Glutamate re-uptake transporters in human brain are the Excitatory Amino Acid Transporters (EAATs) and they play an important role of regulating concentrations of glutamate in the synaptic cleft (for review see (125)). In other mammalian systems, the nomenclature for the EAATs is different. EAAT1 (GLAST) is located on glial cells, while EAAT3 (EAAC1 carrier) is predominantly neuronal (125); EAAT2 (GLT1) can be found on both the glial and neuronal plasma membranes (126-127). Although these transporters are ubiquitously expressed throughout the brain, GLT1 is the major glutamate transporter in the forebrain and GLAST is predominantly expressed in the cerebellum (128-129). Consistent with previous reports (130), we observed GLAST, EAAC1, and GLT1 mostly in the membrane bound fraction but not in the cytosolic fraction of mouse brain extracts (data not shown). None of these transporters (GLAST, EAAC1, and GLT1) showed significantly different levels between APOE-ε3 and APOE-ε4 TR brains (Fig V.3A-C).

We also examined the levels of VGLUT1, a transporter responsible for packaging GLU into presynaptic vesicles. As expected, VGLUT1 was predominantly localized to the membrane bound fraction (Fig. V.3D). Interestingly, the levels of VGLUT1 were significantly higher in APOE-ε4 TR mice compared to APOE-ε3 TR mice (20%, p<0.05) (Fig V.3 E,F, Table1). We also observed low levels of VGLUT1-positive fragments in the cytosolic brain fraction; VGLUT1 fragments were observed in APOE-ε3 TR mice, but not in the APOE-ε4 TR mice (Fig V.3D,E Table 1). The 42 kDa band observed in the TBS brain extracts of APOE-ε3 mice was due to immunoglobulin-related proteins and not a VGLUT1-related fragment (data not shown).
Overall, the data suggest that APOE genotype affects vesicular glutamate within the presynaptic compartments of the glutamatergic synapses.
Figure V.3: VGLUT1 levels are higher in the TBS-X fraction of APOE-ε4 TR mice
4-5 month old male APOE TR mouse brains were homogenized by RIPA and the levels of (A) GLAST, (B) GLT1, and (C) EAAC1 were analyzed by western blot and quantified. These levels were unchanged among the genotypes. (D-F) 4-5 month old APOE TR mouse brains were homogenized by TBS followed by TBS-X via sequential fractionation. (D) VGLUT1 is predominantly in the TBS-X fraction as seen in the representative immunoblot. (E) The immunoblot for VGLUT1 in the TBS-X fraction (top) and TBS fraction (bottom). (F) Quantification for the TBS-X fraction in (E). *, p<0.05. n=4 animals/condition. Error bars expressed as S.E.M.
# Levels in APOE-ε4 TR mice compared to APOE3-ε3 TR mice

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*APOE-ε3 TR mice also have a 42-KDa immuno-positive band observed in the VGLUT1 TBS fraction that is not observed in the APOE-ε4 TR mice.

Table 1: Summary of Figures V.1 and V.3
APOE-ε4 TR mice have significantly higher brain levels of glutamine.

The differences in PAG levels (Fig V.1) and VGLUT1 levels (Fig V.3), suggest that APOE-ε4 TR mice may have altered glutamate metabolism. Therefore, we measured whole brain levels of GLU and two of its downstream products, glutamine (GLN) and gamma-aminobutyric acid (GABA), using high-frequency ¹H NMR spectroscopy in mice at 4-5 months of age (n= 4 per genotype). We chose the 4-5 month age because it was consistent with the timepoint chosen for our immunoblotting analysis. We found that GLU levels were significantly lower in APOE-ε4 TR mice by over 50% compared to APOE-ε3 and APOE-ε2 TR mice (Fig V.4A) (p<0.05). In contrast, the total levels of GLN were significantly higher by 3 fold (p<0.01) in the APOE-ε4 TR mice compared to the APOE-ε3 and APOE-ε2 TR mice (Fig V.4B). Total levels of GABA were not different among the APOE genotypes (Fig V.4C).

We repeated the ¹H NMR analysis in a second cohort of APOE TR mice at 1 year of age. Brain levels of GLU were not significantly different between the APOE-ε4 and APOE-ε3 TR mice, but the APOE-ε2 TR mice still had significantly higher levels of GLU (Fig V.4D) (p<0.05). Again, we did not observe significant differences in levels of GABA among the genotypes (Fig V.4F). Consistent with the 4-5 month results, the levels of GLN were significantly higher (by 123%, p<0.001) in the APOE-ε4 TR mice compared to APOE-ε3 and APOE-ε2 TR mice (Fig V.4E). These consistent data on higher GLN levels in APOE-ε4 TR mice, taken together with the reduction in PAG levels (Fig V.1), suggest that there is decreased metabolism of GLN in APOE-ε4 TR mice compared to controls. This observation does not
exclude the possibility that there is also increased GLN synthesis in APOE-ε4 TR mice compared to controls.
Figure V.4. Glutamine pool sizes are higher in APOE-ε4 TR mice

4-5 month old male APOE TR brains were lyophilized and resuspended in deuterium. $^1$H-NMR was used to measure total metabolite pool sizes for GLU (A), GLN (B), and GABA (C). 1 year old APOE TR brains were lyophilized and resuspended in deuterium. $^1$H-NMR was used to measure total metabolite pool sizes for GLU (D), GLN (E), and GABA (F). At both time points, GLN levels were significantly increased by over two fold in the APOE-ε4 TR mice compared to the other genotypes. *, p <0.05, **, p<0.01, ***, p<0.001 compared to APOE-ε3 TR mice.#, p<0.05, ##, p<0.01 compared to APOE-ε4 TR mice. Error bars expressed as S.E.M. (n=4 animals/genotype/time-point).
APOE-ε4 TR mice have significantly lower brain $^{13}$C incorporation of glutamate

To address the mechanism by which GLN levels are higher in the APOE-ε4 TR mice, we used $^{13}$C NMR spectroscopy. Animals were intraperitoneally injected with [1-$^{13}$C] glucose, and brain tissue was collected 45 minutes later (see Methods Chapter for more detail). This method enables the measurement of $^{13}$C label incorporation from glucose into various metabolites. The data at 1 year and 4.5 months were consistent with each other. APOE-ε2 TR mice showed significantly higher levels of newly-synthesized GLU compared to the other genotypes at both 4-5 months (Fig V.5A), and at 1 year of age (Fig V.5D). These data are consistent with the higher total levels of GLU in APOE-ε2 TR mice observed in Figure V.4. GLU production at 4.5 months and 1 year (Fig V.5A,D) was significantly lower in the APOE-ε4 TR mice compared to the other genotypes (90% compared to APOE-ε2 TR, and 60% compared to APOE-ε3 TR, p<0.001 at 1 year), suggesting that $^{13}$C incorporation of GLU was lower or GLU breakdown was higher in these animals (Fig V.5D).

Turnover rates of GABA (of which GLU is a precursor) were not different among the APOE-ε3 and APOE-ε4 genotypes at 1 year of age (Fig V.5E). The overall incorporation rate of $^{13}$C into GABA is much lower than the $^{13}$C incorporation rate into GLU or GLN (Fig V.5) suggesting that the $^{13}$C incorporation rate into GABA is less efficient perhaps because it is in a different compartment (GABAergic interneurons compared to the GLU-GLN cycle). Other NMR conditions may need to be used to increase GABA enrichment to observe a clear APOE isoform dependent effect. Interestingly, GLN production was not significantly different among the APOE genotypes at 4.5 months (Fig. V.5C) and at 1 year of age (Fig. V.5F), suggesting that
the higher GLN levels in APOE-ε4 TR mice (Fig V.4B,D) were not due to increased GLN synthesis derived from the GLU-GLN cycle. Succinate (a product of the TCA cycle) turnover rates were also not different among the genotypes (Fig.V. 6) suggesting that the lower level of glutamate production was not due to a deficit in entry of glucose-derived $^{13}$C label into the TCA cycle.
Figure V.5. Glutamine incorporation is unchanged among the genotypes
4-5 month old male APOE TR mice were injected i.p. with [1-13C] glucose solution, and brains were extracted 45 minutes later. 13C NMR analysis was used to determine the incorporation of 13C for GLU C4 (A), and GABA C2 (B), and GLN C4 (C). 1 year old APOE TR mice were injected i.p. with [1-13C] glucose solution, and brains were extracted 45 minutes later. 13C NMR analysis was used to determine the incorporation of 13C for GLU C4 (D), and GABA C2 (E), and GLN C4 (F). ###, p<0.001, ##, p<0.01, #, p<0.05 compare to APOE-ε4 TR mice. ***, p<0.001, **, p<0.01, *, p<0.05 compared to APOE-ε3 TR mice. The GLU turnover rate was increased in the APOE-ε2 TR mice and decreased in the APOE-ε4 TR mice when compared to APOE-ε3. (n=4 animals/genotype). Error bars expressed as S.E.M.
Figure V.6. Succinate incorporation is unchanged among the genotypes
1 year old mice were injected i.p. with [1-\textsuperscript{13}C] glucose solution, and brains were extracted 45 minutes later. \textsuperscript{13}C NMR analysis was used to determine the incorporation of \textsuperscript{13}C for succinate C2/C3. n=4 mice/genotype. Error bars expressed as S.E.M.
Discussion

Various findings in humans and APOE knock-in mice indicate that APOE genotype affects normal brain function independent of the accumulation of AD-associated neuropathological changes. We have been able to address this hypothesis by analyzing whether there are effects of APOE genotype in knock-in mice on the metabolism of GLU, the major excitatory amino acid in the brain. We used immunoblots to measure proteins important to GLU metabolic pathways and $^1$H-decoupled $^{13}$C NMR to measure various GLU metabolites. We found several indications that GLU metabolism was altered in APOE-ε4 TR mouse brains, including decreased levels of glutaminase and increased levels of VGLUT1. We also found that $^{13}$C incorporation into GLU is lower in APOE-ε4 TR mice compared to APOE-ε3 and APOE-ε2 TR mice at 4-5 months and at 1 year of age. Human APOE-ε4 carriers have reduced glucose utilization (67, 70, 131) and the APOE-ε4 knock-in mice have a more permeable blood brain barrier (132-133), suggesting that reduced GLU levels observed via $^{13}$C NMR may be related to reduced uptake of glucose by astrocytic endfeet. However, APOE-ε4 TR mice did not have a lower $^{13}$C incorporation into succinate C2/C3 or GLN C4 (Fig. V.6, Fig. V.5C,F). If decreased glucose uptake in APOE-ε4 TR mice were responsible for the reduced $^{13}$C incorporation into GLU C4 observed, then all measured glucose metabolites should have a reduced rate of production.

We also found that total GLN levels were over two fold higher in the APOE-ε4 TR mice compared to APOE-ε3 and APOE-ε2 TR mice; this result was consistent in two independent cohorts of animals (Fig V.4). We examined the various transporters and enzymes involved in the
GLU-GLN cycle. The lower glutaminase levels of APOE-ε4 TR brains (Fig V.1) would result in reduced recycling of GLN back into GLU and thus explain the higher levels of total available GLN. The GLU-GLN cycle is a major pathway for GLU repletion in the brain (121). These findings have suggested that presynaptic mechanisms among the APOE genotypes may account for the observed differences in GLN levels (See model, Fig. V.7). We hypothesize that apoE maybe inhibiting vesicle release either by altering presynaptic membrane lipid composition or altering presynaptic ApoE receptor signaling (this is similar to how we hypothesized apoE changes on postsynaptic density in Chapter I, i.e. through altering postsynaptic membrane lipid composition or altering postsynaptic APOE receptor signaling). Future studies will begin to address this hypothesis.
Figure V.7: The effects of APOE4 on presynaptic markers
A model depicting the alterations in APOE-ε4 TR mice compared to APOE-ε3 TR mice. APOE-ε4 TR mice have increased total GLN (in green) and increased VGLUT1 (in green) but decreased glutaminase (red) compared to APOE-ε3 TR mice. All other aspects of the GLU-GLN cycle are unaffected (such as glutamine synthetase and the other glutamate transporters). We hypothesize that apoE maybe inhibiting vesicle release either by altering synaptic lipid membrane composition or affecting signaling via a pre-synaptic ApoE receptor.
Although the total levels of GLU in APOE-ε4 TR mice were unchanged at 1 year compared to the APOE-ε3 TR mice (Fig 4E), the metabolic flux of $^{13}$C into GLU C4 was lower (Fig V.5), suggesting that APOE-ε4 TR mice may compensate by having a larger pool of readily available GLU. VGLUTs are transporters which use a chloride and proton gradient to shuttle cytoplasmic GLU into pre-synaptic vesicles. The total level of VGLUTs correlates with the amount of GLU stored and released (134-137) at the presynaptic terminal. A recent report showed that APOE-ε4 TR mice on a control diet have higher total VGLUT1 levels compared to APOE-ε3 TR mice (138), consistent with our findings (Fig V.3), suggesting increased vesicular GLU in APOE-ε4 TR mice. The higher VGLUT1 in APOE-ε4 TR mice would thus compensate for the reduced GLU turnover by increasing the quantal content of GLU in synaptic vesicles (See model, Fig V.7). Although VGLUT1 is predominately expressed on synaptic vesicles, when the vesicles fuse to the plasma membrane and release GLU, VGLUT1 is also present on the plasma membrane. Plasma membrane VGLUT1 is involved in phosphate transport (128). High concentrations of phosphate are needed to activate glutaminase, and VGLUT1 on the plasma membrane correlates with an increase in glutaminase activity (128). Thus, in APOE-ε4 TR mice, higher VGLUT1 levels at the plasma membrane may be a compensatory mechanism for the observed reduction in glutaminase levels.

In contrast to the membrane fractions, we observed decreases in VGLUT1 levels in the cytoplasmic fractions of APOE-ε4 TR mice (Fig V.3E). The presence of full-length VGLUT1 and VGLUT1 fragments in cytoplasmic extracts may reflect turnover of membrane-bound VGLUT1, although little is known about VGLUT1 metabolism (139). Recently, it has been
reported that VGLUT1 levels on synaptic vesicles can be impacted by the diurnal cycle (140-141), however, the precise mechanism regulating VGLUT1 levels are unknown. The observation of lower levels of VGLUT1 immunoreactive bands in APOE-ε4 mice may indicate that they have reduced rates of VGLUT1 turnover. The apoE4 protein has been observed to reduce recycling of endocytic vesicles (142-143), and it may similarly impact VGLUT1 recycling in synaptic vesicles.

Here, we focused on elements of the GLU-GLN cycle, but GLU can also be derived from other sources in the brain. For example, leucine, which is transported across the blood brain barrier, can be transaminated into GLU (144). It is possible that the leaky blood brain barrier of APOE-ε4 knock-in mice may impact leucine trafficking into the brain, which subsequently could impact GLU metabolism.

Our observed alterations in GLU metabolism support earlier studies showing synaptic alterations in APOE-ε4 TR mice (35-36, 110, 145). They may also relate to a recent study that found seizure activity as well as abnormal EEG cortical activity in APOE-ε4 TR mice (41). Alterations in the precise balance of excitatory and inhibitory neurotransmission could underlie the susceptibility of APOE-ε4 TR mice to seizures. This recent finding in mice correlates with another recent study, which has found that healthy human APOE-ε4 carriers have abnormal EEG activity following hyperventilation (146).

Understanding the biochemical variations that correlate with APOE genotype in these mouse models could allow advancement for clinical approaches in APOE-ε4 carriers. There are reports of aberrant expression of glutaminase as well as glutamine synthesis and glutamate dehydrogenase (which converts α-ketoglutarate to glutamate) in AD brains compared to controls.
Unfortunately, in these studies, it is impossible to delineate whether these changes were present prior to onset of AD. These APOE TR mice allow us to examine such changes independent of the AD pathology.

Due to the limitations of NMR sensitivity, we were using whole brain extracts enriched with $^{13}$C label to achieve our signal, therefore the effects that we observe have been averaged across brain regions. Future studies will begin to elucidate the brain specific region effects of APOE-$\varepsilon$4 TR compared to the other genotypes. Such studies would be interesting, especially with the conflicting reports of LTP abnormalities in APOE-$\varepsilon$4 TR mice: LTP is enhanced in the CA1 region of the hippocampus (36) but reduced in the dentate gyrus (145).

Here, we have defined several presynaptic effects of APOE genotypes. However, we and others have found that APOE genotype also affects post-synaptic measures. APOE-$\varepsilon$4 correlates with decreased dendritic spines in both mice (35, 110) and humans (93). Primary neurons treated with recombinant apoE4 recombinant protein have reduced surface level expression of glutamate receptors (AMPA and NMDA subunits) compared to control and apoE3 treated neurons (142). Future in vitro studies will address whether apoE receptors are involved in modulating the presynaptic (glutamate synthesis, VGLUT1 levels, glutaminase levels) and postsynaptic (GLU trafficking, dendritic spines) effects of APOE4, to begin elucidating the mechanism of these isoform specific effects. The various in vivo measures have thus allowed us to focus future in vitro analyses on specific neurotransmission pathways and test whether the pre- and post-synaptic changes in APOE-$\varepsilon$4 TR mice are independent or related events.

A potential approach to manipulating apoE isoform effects is through diet. APOE-$\varepsilon$4 TR mice fed on a diet high in DHA content resulted in lower levels of VGLUT1, abolishing the
effects of APOE-ε4 (138). These results indicate that not only does diet interact with APOE genotype to affect the lifecycle of GLU, but also that preventative measures may allow reduction of the differences in APOE-ε4 and APOE-ε3 TR genotypes. The various measures of proteins and metabolites from this study could act as biomarkers of AD risk. ApoE is a cholesterol transporter and essential to maintaining lipid and cholesterol homeostasis in the membrane. Although total membrane composition of cholesterol and lipids is not strikingly different in these animal models (138), it is quite possible that the membrane composition at the synaptic vesicle is altered. Thus, AD preventative treatments of normal mice could be monitored using these new \emph{in vivo} markers of APOE genotype.

Interestingly, APOE-ε2 TR mice are markedly different from the APOE-ε3 and APOE-ε4 TR mice, potentially relating to the decreased risk of AD associated with APOE-ε2 (149). We observed some opposite effects of APOE-ε2 compared to APOE-ε4 in these mice, such as higher $^{13}$C label incorporation into GLU in the APOE-ε2 TR animals (Fig 5A, Table 1), as well as higher levels of brain GLU (Figure 4). As in humans, APOE-ε2 is associated with type III hyperlipoproteinemia in these mice (149), resulting in atherosclerotic plaques and over double the cholesterol and triglyceride levels of the other APOE genotypes. The observed differences in brain GLU flux or level of $^{13}$C incorporation (E2>E3>E4) could be related to differences in lipid metabolism or could reflect differences in risk of neurodegeneration by APOE genotype.

\textbf{Conclusion:} In this work, using a variety of measures, we have shown that APOE genotype modulates glutamate metabolism in a mouse model displaying normal expression of human APOE alleles. In the APOE-ε4 TR mice, glutamate level was low while glutamine levels
were high; in addition, glutaminase levels were low and VGLUT1 levels were high. These various factors may contribute to the increased risk of neurodegeneration associated with APOE-ε4, and could thus act as surrogate markers for AD risk.
Chapter VI: Determining whether APOE genotype differentially affects ultrastructural components at pre-synaptic terminals

In the previous chapter, we observed that glutamine (GLN) levels were over 2 fold higher in APOE-ε4 TR mouse brains compared to the other APOE genotypes (Fig V.4), but the functional implications are unknown. We also observed that glutaminase, the enzyme responsible for converting GLN to GLU was reduced by ~20% in the APOE-ε4 TR mice (Fig V.1). GLN is an abundant, naturally occurring, non-essential amino acid that can cross the blood brain barrier (150-151). Metabolically, GLN can be utilized in numerous ways. For example GLN can act as a nitrogen donor for protein synthesis (152), a carbon donor to the TCA cycle (153-154), as well as a substrate for gluconeogenesis (155), a substrate for synthesis of the neurotransmitters glutamate (GLU) and GABA ((156), and a substrate for glutathione, an important cellular antioxidant (157). Moreover, oxidation of GLN, via glutaminolysis, can be used as an alternative energy source to drive mitochondrial ATP (158-159). Indeed, many cells require GLN for survival, which have many re-classifying GLN as a “conditionally essential” amino acid (160) for the body.

However, evidence suggests that too much GLN in the brain can also be neurotoxic. Indirect evidence of GLN toxicity came from observations that patients diagnosed with hepatic encephalopathy (HE) have elevated levels of GLN CSF from 2-7 times the normal range (161). HE is encephalopathy causally related to liver damage, and the subsequent neuro-inflammation is traditionally believed to result from excessive amounts of ammonia crossing the blood brain barrier. Because ammonia can react with GLU to form GLN, the observed increase in GLN CSF
content was initially thought to be a nontoxic byproduct of ammonia neutralization in HE patients. However, HE symptoms, specifically brain swelling, can be alleviated by methionine sulfoximine (MSO) treatment (161). MSO is a glutamine synthetase inhibitor, which thereby inhibits GLN synthesis and wouldn’t prevent ammonia detoxification. The successful course of MSO treatment suggests that the increase in GLN, not ammonia, may be the cause of neuroinflammation. This idea is further strengthened by studies showing that treating glial/neuronal mixed cultures with high levels of GLN causes mitochondrial swelling and oxidative stress (162). GLN can act as a “Trojan Horse”, as it is easily transported into mitochondria where it can be hydrolyzed to ammonia and glutamate (163). Indeed, the GLN induced mitochondrial stress can be alleviated by inhibiting glutaminase, the enzyme responsible for hydrolyzing GLN to GLU and ammonia, in vitro (162). The generation of high levels of ammonia inside the mitochondria can cause mitochondrial permability transition, mitochondrial swelling and oxidative damage (164-165).

Mitochondria efficiency at the synapse impacts synaptic vesicle cycling as this process involves numerous ATP consuming steps and is calcium concentration dependent (166). In the previous chapter, we had reported a ~ 20% increase in membrane bound vesicular glutamate transporter I (VGLUT1) in APOE-ε4 TR mice compared to APOE-ε3 TR mice. VGLUTs are transporters which use a chloride and proton gradient to shuttle cytoplasmic GLU into presynaptic vesicles and total level of VGLUTs correlates with the amount of GLU stored and released (134-137) at the presynaptic terminal. The increase in VGLUT1 observed in the APOE-ε4 TR mice could imply that there is an increase in overall excitatory presynaptic vesicles or that the amount of VGLUT1 was increased per vesicles.
Because we had observed a substantially higher levels (over 2 fold) in GLN levels in the APOE-ε4 TR mice compared to the other genotypes, we wanted to know whether these levels were toxic enough to impact mitochondrial morphology. Specifically, we hypothesized that APOE-ε4 mice would have larger, swollen mitochondria. To test this, we used transmission electron microscopy, and examined the morphology of mitochondria randomly imaged in cortical layers II/III above the dorsal hippocampus in 12 month old female ApoE-ε3 and APOE-ε4 TR mice. Because we had observed the increases in VGLUT1, we also used transmission electron microscopy in these animals to ask whether this increase in VGLUT1 was associated with an overall increase in presynaptic vesicles at excitatory synapses. A presynaptic excitatory terminal was defined as one with an asymmetric synaptic cleft, specifically one where there was a prominent postsynaptic density and spherical synaptic vesicles on the pre-synaptic side.

Results

When we imaged the mitochondria (120 from the ε3 and 140 from the ε4 genotypes) we observed four distinct mitochondrial morphologies: healthy, degenerating, vacuolar budding, and immature (Fig VI.1A). Healthy mitochondria were defined as mitochondria with intact membranes and compact continuous cristae that cover the majority of the mitochondrial area. Degenerating mitochondria were defined as swollen mitochondria with fragmented cristae. Budding was defined as mitochondria, which had a cytoplasmic vacuolar budding pushing the mitochondrial membrane out but intact cristae were still observed. Immature types were defined as mitochondria with intact membranes and few intact observable cristae. The majority, around 57%, of the imaged mitochondria in both groups were categorized as healthy. 17% (ε3) and 25%
(ε4) of the mitochondria imaged were classified as degenerating while 18% (ε3) and 11% (ε4) as vacuolar budding. Lastly, 7% (ε3) and 5% (ε4) were classified as immature. Using a Chi-squared test, we observed no significant difference in the percentages of mitochondrial types between the two genotypes (Fig VI.1B).

Mitochondrial size was measured as the width across the mitochondria from its shortest diameter in the cross-sectional images collected. The average mitochondrial size was similar among the genotypes at around 430 nm (Fig VI.1C). Because the range of mitochondrial sizes varied from 100 to 900 nm, we also looked at the distribution patterns, to determine whether there were different populations of mitochondria that varied among the genotypes. However, the distribution plots were similar among the two APOE genotypes (Fig VI.1D), indicating that the distribution in overall mitochondrial size was not dependent on APOE genotype.
Figure VI.1. APOE-ε3 and APOE-ε4 TR mice have similar mitochondrial morphology in cortical layers II/III. Images of mitochondria in cortical layers II/III above the dorsal hippocampus were taken of female 12 month old APOE-ε3 and APOE-ε4 TR mice at 40,000x magnification with a Transmission Electron Microscope (n=3 animals/genotype, 120 total mitochondria from the ε3 and 140 total from the ε4 genotypes) A. Representative images of the types of mitochondria that were seen: Healthy (upper left panel), degenerating (upper right panel), immature (lower left panel), and vacuolar budding (lower right panel). Black bar represents 250 nm. B. Quantification of the categorization of mitochondria observed did not differ among the genotypes. C. Average mitochondrial diameter width, measured as the shortest diameter across in nm, was similar among the genotypes. Error bars represent S.E.M. D. Histogram profile of mitochondrial size for ε3 (triangle) and ε4 (square) genotypes was similar. Mitochondrial width ranged from 100 to 900 nm, with the majority of mitochondria being 200-500 nm wide.
We observed that the size, shape and type of mitochondria varied depending on where it was localized in the cell. For example, degenerating mitochondria were more often observed in the soma or in axons but not in the pre-synaptic boutons of axonal terminals or in the post-synaptic dendritic compartments. Because we observed a huge range in mitochondrial size and shape depending on location, and our work has specifically observed APOE dependent changes pre-synaptically (Chapter V), we wondered whether the subset of mitochondria in pre-synaptic terminals might morphologically vary between the two APOE genotypes. Therefore, we imaged ~60-70 mitochondria per genotype that were present in presynaptic excitatory terminals. A presynaptic excitatory terminal was defined as one with an asymmetric synaptic cleft, specifically one where there was a prominent postsynaptic density and spherical synaptic vesicles on the pre-synaptic side (For representative image see Fig VI.2A). The majority of mitochondria ranged from 100 to 300 nm in width, and the distribution of mitochondrial size was similar among the two APOE genotypes (Fig VI.2B). However, on average, the APOE-ε4 mitochondria were significantly smaller in width by about 10% (p<0.05) compared to the APOE-εε TR mice (Fig VI.2C).
Figure VI.2. APOE-ε4 TR mice have a decrease in mitochondrial size at excitatory presynaptic terminals compared to APOE-ε3 TR mice. Mitochondria were imaged at 40,000x magnification from 12 month old APOE TR females in cortical layers II/III (n=3 animals/genotype, ~60-70 total mitochondria images per genotype). A. A representative image of a mitochondria at a presynaptic terminal. The arrow points to the mitochondria. The black bar represent 250 nm. B. Distribution plots of ε3 (triangle) and ε4 (square) mitochondria as a function of size. Mitochondrial width was measured as the smallest diameter across. There were no significant differences in the distribution of mitochondrial size between the genotypes. C. Quantification of the average mitochondrial size. ApoE-ε4 TR mice have a 10% decrease in mitochondria diameter width compared to APOE-ε3 TR mice. *, p < 0.05. Error bars represent S.E.M.
Because we had observed a 20% increase in total VGLUT1 levels (Chapter V) in APOE-ε4 TR compared to the other genotypes, we wanted to know whether the increase in VGLUT1 indicated that there were an increase in total number of presynaptic vesicles for APOE-ε4 TR mice, or whether it meant that the amount of VGLUT1 per vesicle was increased. To test the former hypothesis, we imaged ~70-80 excitatory presynaptic terminals per genotype and counted the number of spherical vesicles divided by the surface area of the pre-synaptic terminal observed, subtracting the mitochondria area if it was present. An excitatory pre-synaptic terminal was defined as an asymmetric synapse, one in which there was a predominate post-synaptic density and spherical vesicles (for representative image, see above, Fig VI.2A). The vesicle density was similar between the two APOE genotypes (Fig VI.3A). However, the distribution of synaptic vesicle density was different among the two genotypes (Fig VI.3B). Specifically, the APOE-ε3 TR mice seem to have two distinct types of pre-synaptic terminals, one with few synaptic vesicles (density ranging from .00025 to .00045 vesicles/nm²) and another population of greater synaptic vesicles (density ranging from .0005 to .0075 vesicles/nm²). The APOE-ε4 mice did not display this phenotypic distribution but more of a Gaussian distribution. For example, 30% of the ε3 vesicles were in the .005-.0075 range, while only 11% of the ε4 vesicles were. Future studies in electrophysiology will attempt to sort whether these differences also translate to altered presynaptic release of vesicles among the APOE genotypes.
Figure VI.3. Overall Synaptic Vesicle Density in APOE TR mice at excitatory synapses in the cortex. Asymmetric presynaptic terminals were imaged in the cortex of 12 month old APOE-ε3 and APOE-ε4 TR females mice, 70-80 images per genotype (3 animals per group). A. Synaptic vesicle density was calculated by determining the overall number of vesicles and dividing by the cross-sectional area of the pre-synaptic terminal imaged. The average synaptic density was similar between the APOE genotypes. Error bars are S.E.M. B. Distribution profiles of the synaptic vesicle density ε3 (triangles) and ε4 (squares) APOE genotypes.
Discussion

We used transmission electron microscopy to examine overall ultra-structural changes to mitochondrial type and size as well as potential changes at the pre-synaptic terminal. We observed no differences overall in mitochondrial size (Fig VI.1), but found a 10% decrease in mitochondrial diameter at pre-synaptic terminals of APOE-ε4 compared to APOE-ε3 TR mice (Fig VI.2). This result was counter to our hypothesis that excess GLN in the APOE-ε4 TR mice would result in increases in swelling and damaged mitochondria compared to APOE-ε3 TR mice. However, to properly test whether GLN could indirectly cause toxicity by its hydrolyzation back into GLU and ammonia (via the “Trojan Horse” hypothesis), we would need to establish whether ammonia levels are increased in mitochondrial preps from pre-synaptosomal fractionations in the APOE-ε4 TR mice. It is possible, that lower levels of glutaminase, the enzyme responsible for hydrolization of GLN at mitochondria, are protecting APOE-ε4 TR mitochondria from the resulting increase in ammonia and subsequent swelling of mitochondria.

Mitochondrial surface area is believed to impact calcium and ATP buffering (166-167). The 10% decrease in the cross-sectional mitochondrial surface area observed in APOE-ε4 TR mice could imply reduced mitochondrial efficiency. Future studies, where we isolate mitochondria from synapses of APOE-ε3 and APOE-ε4 TR mice can test for ATPase activity to determine whether this is the case. Interestingly, two recent studies support the hypothesis that APOE-ε4 genotype impacts mitochondria efficiency. Specifically, a recent proteomics study reported that APOE-ε4 TR mice have reduced levels of Complex I subunits compared to APOE-ε3 TR mice (168). Another study examined postmortem tissue and reported that young APOE-ε4
human carriers, without any amyloid pathology, had lower levels of cytochrome oxidase (the Complex IV component of the electron transport chain) compared to non-APOE-ε4 carriers (169).

We also used transmission electron microscopy to determine whether synaptic vesicle density was altered among the APOE genotypes. In the previous chapter, we observed an increase of VGLUT1, the transporter responsible for packaging GLU into vesicles in cortical regions (Fig IV.3). In our preliminary studies, we observed no difference in the average synaptic vesicle density (Fig V.3A). Caveats to this preliminary study are that we did not examine vesicle size, or distinguish between synaptic vesicle types. For example, it is possible that APOE-ε4 TR mice have larger synaptic vesicles compare to APOE-ε3 TR mice. Moreover, some synaptic vesicles displayed a dense halo indicative of clatherin mediated processes (170), while others did not. Categorizing vesicles by type and number would better distinguish the potential differences between APOE genotypes on synaptic vesicles.

In our initial examination however, the data suggest that the increase in VGLUT1 protein levels is not due to increased synaptic vesicles but rather increased number of VGLUT1 per vesicle. To test this, we will use immuno-gold VGLUT1 labeling and assess the intensity of VGLUT1 staining per vesicle between the APOE genotypes.

Interestingly, the distributions of overall synaptic vesicle density qualitatively differed between the APOE genotypes (Fig VI.3B). Specifically, the APOE- ε3 mice had two distinct types of synapses (a smaller pool and a larger pool), which the APOE-ε4 mice did not have this distinction. Future studies utilizing electrophysiology can assess whether these differences
translate to qualitative differences in synaptic signaling using slice cultures. For example, we may observe that the overall changes in mEPSCs is similar between the genotypes, but that the distribution of the frequency in mEPSCs mirrors that observed in synaptic vesicle distribution.

We can also move to an \textit{in vitro} system to measure the exocytosis rates of vesicles as briefly described in (171). Briefly, primary neurons from APOE-\(\varepsilon\)3 and APOE-\(\varepsilon\)4 neurons can be transfected with super-ecliptic synaptopHluorin (SPH), which is a SNARE protein fused to a pH sensitive GFP. SNARE proteins are involved in the docking of vesicles to the membrane allowing for neurotransmitter release (171-172). As soon as the synaptic vesicle fuses to the membrane, the sudden rise in pH will potentiate the fluorescence of SPH, which can be visualized via a live cell imaging microscope. Various experimental set-ups can utilize different depolarization protocols (i.e., 50 mM KCL) to elicit vesicle release.

\textbf{Conclusions:} We conducted an investigation in the ultra-structural integrity of mitochondria of APOE TR mice. We found that there was a 10\% decrease in mitochondrial diameter width in cross-sectional images in excitatory pre-synaptic terminals of APOE-\(\varepsilon\)4 TR mice compared to APOE-\(\varepsilon\)3 TR mice. Future studies will determine whether this is indicative of reduced mitochondrial efficiency. We also examined synaptic vesicle density at excitatory presynaptic terminals, and found no quantitative differences between the APOE genotypes, although there are indications that there are qualitative differences in synaptic vesicle distribution profiles. Future studies utilizing electrophysiology will explore this area further.
Chapter VII: Glutamine as a CSF biomarker of APOE-associated AD Risk

In chapter III, we reported that cortical neurons from APOE-ε4 mice have significantly reduced dendritic arborizations and dendritic spine densities (also see (110)). These findings suggested to us that APOE-ε4 genotype leads to simplified synaptic connections early in life, which could contribute to susceptibility to damages later in life. Because dendritic spines are post-synaptic sites of glutamatergic transmission, we then tested whether APOE genotype altered glutamatergic signaling in mouse brains. Using NMR assays, which allowed us to measure metabolites such as glutamate, glutamine (GLN), and GABA, we found that the levels of GLN were significantly higher in APOE-ε4 animals in both cohorts of mice that were analyzed, 4-5 month old and 1 year old (Chapter V, (173)).

Our hypothesis is that the APOE-ε4 allele affects brain activities well before AD pathogenesis occurs and that biomarkers of APOE-associated risk could be followed in preventative therapeutic approaches in control individuals. If true, would expect that humans with APOE-ε4 would have decreased spine density (Chapter III) and brain levels of GLN would be increased (Chapter V).

A study examining post-mortem tissue from control patients that were negative for amyloid staining, found that APOE-ε4 carriers had a 15% reduction in spine density (93), although this study was small (examining 10 total control samples), it gives face validity to using our animal models for biomarkers. However, spine density is not an ideal biomarker, as it is difficult to be measure and cannot be assessed in live individuals. We sought to measure another
potential biomarker, specifically we wanted to test whether significant differences in GLN in the brains of APOE-ε4 mice will correlate with increased levels of GLN in the cerebrospinal fluid (CSF) of humans carrying the APOE-ε4 allele.

Results

To address whether GLN levels were increased in APOE-ε4 carriers, we initiated a pilot study of ante-mortem human CSF, to test whether there is a strong difference in these measures as was observed in mouse brains. From the Washington University Alzheimer’s Disease Research Center, we received a total of 20 samples from the three most common APOE genotypes (6 ε2/ε3, 7 ε3/ε3, 7 ε3/ε4). These individuals were matched for age (between 50-60 years old), and deemed cognitively normal as assessed by the clinical dementia rating (CDR) scores. We measured levels of glutamate and glutamine using samples spiked with deuterated GLN and GLU, obtaining reproducible standard curves (Fig. VII.1A,B). Our CSF levels showed concentrations consistent with published levels of each metabolite from large numbers of ante-mortem tissue (174). The GLN levels ranged from 772.5 to 1208.2 microMolar while GLU levels ranged from 13 to 23 microMolar. For GLU, there were no differences observed across APOE genotypes (Fig. VII.1C). For GLN, we observed higher levels in CSF from the 7 APOE ε3/ε4 individuals; these higher levels were significantly greater than those in 6 APOE−ε2/ε3 individuals (p<0.05), and not quite significantly higher than the 7 APOE−ε3/ε3 individuals (p<0.09) (Fig. VII.1D). These findings provide support for our earlier hypothesis that glutamine levels are increased in brains of individuals with APOE−ε4.
Figure VII.1. Glutamate and Glutamine CSF values stratified by APOE genotype. A. Glutamate standards (grey symbols) defined a standard curve and glutamate levels from 20 different CSF samples done in triplicates (black symbols) were determined. B. Glutamine standards (grey symbols defined a standard curve and glutamine levels from CSF samples (black symbols) were determined. C. Glutamate CSF levels were separated by APOE genotype. D. Glutamine CSF levels were separated by APOE genotype. Student’s one-tailed t-tests showed that APOE−ε3/ε4 CSF had significantly higher glutamine than APOE−ε2/ε3 CSF (p<0.05), and a trend toward higher levels than APOE ε3/ε3 CSF (p < 0.09).
Discussion:

We analyzed CSF samples from cognitively normal individuals, drawn from the three most common APOE genotypes: APOE ε2/ε3, APOE ε3/ε3, and APOE ε3/ε4. We found that APOE ε4/ε3 carriers had significantly increased levels of GLN compared to ε2/ε3 carriers, and a trend toward an increase compared to ε3/ε3 carriers. Please note that the appropriate statistical analysis would be the 1 way ANOVA, which did not reach statistical significance. However, as this is a small n number, the results are still encouraging to continue with a larger sample. Our hypothesis would be that those ε3/ε3 carriers which had levels of GLN at the higher end of the spectrum would be at increased risk of getting AD. To extend these studies, recently we received samples that will allow us to increase our sample size to 20 individuals of each of the common genotypes (εX/ε3, previously we had examined 6-7 samples each), and are also comparing these values to the rare APOE−ε4/ε4 genotype. This increase would allow us to determine whether APOE−ε3/ε4 individuals have significantly higher levels of glutamine in their CSF compared to APOE−ε3/ε3, and whether there is increased variance in the measures of this metabolite in individuals at higher risk of AD. Moreover, we would be able to ask whether carriers homozygous for ε4, who are at even greater risk for AD, also have greater levels of GLN. Note that there was a range of GLN concentrations observed. For example, the range for the non-ε4 genotypes ranged from 772 to 1094 µM, while ε4 heterozygote carriers had GLN levels ranging from 831 to 1208 µM. There were 2 of these ε4 heterozygote carriers on the lower spectrum. We would hypothesize that they would be at decreased risk of getting AD compared to the other ε4 carriers.
If we do find a significant correlation between GLN levels and APOE status, we can then ask at what age the GLN differences occur. For example, a recent study provides evidence that AD related changes begin occurring 2 decades before actual diagnosis (7), which implies that some of these CSF changes in our 50-60 year cohort may be due to the disease process already beginning, despite appearing cognitively normal. By examining younger ages (for example, controls in their 20’s), one can better delineate APOE effects from AD related changes. Moreover, we will also follow these donors as they age, to determine whether those with higher levels of GLN do indeed get diagnosed with AD later on in life. If this is indeed the case, than GLN will be a novel biomarker that can be used as an outcome measure in preventative and clinical trials.
Chapter VIII: Overall Discussion

Alzheimer’s disease (AD) is the 6th leading cause of death in the US. Unlike the other leading causes of death, such as cardiovascular diseases, AD cannot be effectively slowed, prevented or treated. The US spends over 200 billion dollars annually to care for those afflicted with the disease. However, since 2000, the mortality rates of AD related deaths have risen by 66% while the rates for the other leading causes of deaths have been reduced (175). According to the National Institute of Aging website, there are currently 90 AD clinical trials in place, but over the past years countless numbers of these promising clinical trials have failed despite success in pre-clinical AD animal models (176). One of the reasons for these failures has been attributed to these human trials occurring at late phases of the disease, with many believing that they should be started earlier in the AD pathogenesis cascade (176). This hypothesis is strengthened by the observation that AD related biological changes occur at least 2 decades before the patient is presented in the clinic with outward symptoms for treatment (7). These findings highlight the need for preventative treatments which can halt the biological cascade before it has begun. To address this issue, three major clinical trials are taking place that are focused on treating familial AD patients, who based on their mutations are guaranteed to be afflicted with AD later on in life, but who are currently asymptomatic (176). If these trials are successful, they will prove the “too little too late” hypothesis for why preclinical treatments in AD mouse models are not translating to the human population.
These trials, even if successful, will not be able to address the majority of AD cases, which are sporadic (with no clear deterministic gene associated with its cause). Therefore, new viable biomarkers are needed to assess when someone is in the early stages of the disease, to determine whether treatment is needed as well as for assessing better prevention strategies.

These attempts are partially analogous to the strides made in determining the cholesterol-cardiovascular disease link. In the 1950’s, when there was a clear established link between cholesterol and cardiovascular disease, researchers set out to find a way to reduce the risk. In 1976, Akiko Endo developed the first statin, a cholesterol synthesis inhibitor. With the use of statins and lifestyle changes to reduce cholesterol levels, the risk of heart disease has dropped by 40% (177). Our goal is to find such a biomarker for sporadic AD cases, so that better preventative strategies can be developed than those currently in place.

The largest genetic risk factor for AD is APOE, with the ε4 allele increasing one’s risk (17-18) and the ε2 allele decreasing one’s risk for the disease (23). Accumulating evidence suggests that APOE impacts normal brain function, independent of AD pathology (67-68, 178). Therefore, we wanted to characterize the differences between the at risk group for AD (the ε4 carriers) and the not-at risk group (non-ε4 carriers), to determine whether any new biomarkers could be discovered that then could be used in determining whether someone is at risk for AD.

To do this, we utilized APOE Targeted Replacement (TR) mice. These animals express the human APOE alleles (APOE-ε2, APOE-ε3, or APOE-ε4) under the mouse APOE promoter, and do not develop the plaques and tangles diagnostic of AD (34). We focused specifically on any changes at the synapse, because synaptic loss is the strongest correlate to cognitive decline in AD (179).
Using these mouse models, we found that cortical neurons from APOE-ε4 mice have significantly reduced dendritic arborizations and dendritic spine densities compared to APOE-ε3 and APOE-ε2 TR mice at 4 wks, 3 months and 1 year time-points (Chapter III, (110)) with the effects increasing as a function of age. This surprising finding suggested to us that APOE-ε4 may lead to simplified synaptic connections early in life, which could in turn contribute to susceptibility to damages later in life. A study examining post-mortem tissue of human APOE carriers (93), although with a small sample size (n=10 total control samples) supports these results, finding that human ε4 carriers asymptomatic of AD had about a 15% reduction in spine density compared to ε4-negative carriers. Moreover, these studies provide support towards the APOE knock-in mouse models having face validity.

How do apoE isoforms differentially regulate spine density?

APOE-ε4 knock-in mice have lower levels of apoE protein observed in brain and plasma compared to APOE-ε3 knock-in mice, despite having similar transcription rates of APOE (50-51). We have also confirmed these findings in our cohort, with the apoE4 proteins levels being reduced by 30% compared to the other APOE genotypes (data not shown). These lower levels of apoE4 may be due the amino acid substitution at the 112 position, resulting in a molten globule state that is associated with reduced stability (42). A recent study has found that healthy apoE4 human carriers also have reduced levels of apoE in their CSF (29). Thus, the apoE4 associated
effects towards AD may be due to loss of function of apoE. Indeed, APOE deficient mice have reduced spine density similar to those observed in APOE-ε4 knock in mice (Chapter III, (110)).

One of apoE’s roles is to provide neurons with high density lipid particles, which are necessary for dendritic spine remodeling and the formation of mature synapses (98). If apoE4 is deficient in providing the high density lipid particles, it may lead to a reduced number of dendritic spines. Studies in APOE deficient mice have shown reduced levels of brain phospholipids, a major component of synaptic plasma membranes (180-181). Interestingly, a recent study utilizing lipidomic profiling in the brains of APOE-ε4 TR mice also reveal altered levels of phospholipids (for example, levels of the phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine are reduced) compared to APOE-ε3 TR mice (138), confirming the loss of function hypothesis. Interestingly, these phospholipid levels could be modulated by diet, such as one supplemented with fish oil (DHA) or reduced cholesterol (138).

The differences in phospholipids observed in the APOE TR mice were slight, for example a ~12% reduction in phosphatidylserine levels in the APOE-ε4 compared to APOE-ε3 TR mice (138). Much of the brain lipid is incorporated into myelin and ApoE may affect only a subset of CNS membranes. It is possible that isolation of synaptic plasma membranes and lipidomic analysis may demonstrate greater differences between the APOE genotypes.

If APOE-ε4 is indeed acting as a loss of function, then treatments which raise apoE levels may make an APOE-ε4 TR mouse more like an APOE-ε3 TR mouse. For example, one could treat with TO91317, an LXR agonist which increases ApoE levels. However, because this drug displays liver toxicity (182), the treatment can only be limited and would not be an ideal therapeutic. Another potential drug to try would be the FDA approved Bexarotene, an RXR
agonist, which can complex with LXR and has been shown to increase total levels of apoE (183). Because this drug is already FDA approved, if this drug was found to be successful in making an APOE-ε4 mouse more like an APOE-ε3 mouse, this could be a drug that would easily translate to clinical preventative trials.

However, it is also possible that increasing apoE4 levels could make the APOE-ε4 TR mouse even more different from APOE-ε3 TR mice. Due to the altered tertiary structure of apoE4, from the Arg112, apoE4 is more susceptible to fragmentation (46). Some believe that these fragments are toxic, and cause apoE related inflammation. Therefore, it is possible, that ApoE4 may have a toxic gain of function which would be enhanced due to apoE agonist treatments. Interestingly, both APOE enhancing (Bexarotene) and APOE reducing (APOE immunotherapy) strategies are currently being utilized in AD animal models (183-184). Both of these studies are using amyloid load as a measure of successful treatment. However, because apoE may be involved in many different functions, it is possible that other outcome measures should also be analyzed in these therapies, such as behavioral outcomes on spatial memory or total GLN levels, to understand what role apoE levels has on these functions. This further illustrates how little is known about apoE’s exact role in the disease. It is quite possible that apoE may be involved in various aspects of the AD biological cascade and that treatment for AD may vary depending on what stage of the disease the patient is in.

Another study which suggests that ApoE4 has altered function, not just a loss of function, is through altered rates of high density lipid recycling. This study demonstrated that apoE4 impairs high density lipid recycling in Hepatoma cells using radioactive and immunofluorescence assays compared to apoE3 (143). Specifically, apoE3 readily undergoes
retro-endocytosis, while apoE4 remains trapped intracellularly for an extended period of time. ApoE binds to a family of transmembrane proteins that mediate endocytosis of ligands and are then recycled back to the cell surface (52). These receptors are involved in neurite outgrowth and long term potentiation (96). If apoE4 impairs vesicle recycling to the cell surface, than this may also reduce the cell surface levels of ApoE receptors. Indeed, ApoE4 treatment on neuronal cultures reduced surface level expression of the ApoER2 receptor, by trapping it in vesicles as it is being recycled to the surface (142). Therefore, apoE isoforms may differentially regulate spine density through modulating ApoE receptor levels and subsequent internal signaling.

To test whether ApoER2 could be involved in dendritic spine formation, we used primary hippocampal cultures and demonstrated that over-expression of ApoEr2 in neuronal cultures enhanced dendritic spine density (Chapter IV). We also demonstrated that modulating the surface levels of ApoEr2, impacted dendritic spine density, by altering expression levels of cytoplasmic adaptor proteins such as X11, which reduced ApoEr2 surface levels, or PSD95, which increased ApoEr2 surface levels (Chapter IV). To further these studies, we should repeat the over-expression ApoEr2 studies in primary neurons, and treat with lipidated-apoE4, lipidated-apoE3, or control. We would hypothesize that apoE4 would reduce ApoEr2 levels compared to control and apoE3 treatment, thereby reducing the observed ApoEr2 dependent increase in dendritic spine formation.

The study which demonstrated that apoE4 impairs ApoEr2 mediated recycling, also demonstrated that primary neurons treated with recombinant apoE4 have reduced surface level expression of glutamate receptors (AMPA and NMDA) compared to control treatment (142). Although we did not observe any changes in total levels of glutamate receptors in adult ApoE TR
mice (Appendix A), we did not examine whether surface levels were altered. We could attempt to do surface immunostaining on tissue using non-premeablizing conditions, to determine what the levels are of surface plasma membrane bound glutamate receptors in APOE TR sections in vivo. Moreover, we could use electrophysiology to study whether there are alterations to either AMPA or NMDA related signaling in hippocampal slices derived from APOE TR mice. For example, one study reported that reelin enhancement of glutamatergic signaling is inhibited in APOE-ε4 TR hippocampal slices but not in slices derived from the other genotypes (142). Reelin enhancement of glutamatergic signaling is mediated through ApoEr2, which can interact with NMDA receptor subunits (58). If apoE4 reduces ApoEr2 levels at the surface, than reelin enhancement of NMDA signaling is also minimized.

**How do apoE isoforms differentially regulate pre-synaptic terminal composition?**

Because we had found differences in the morphology of post-synaptic excitatory sites (ie. dendritic spines), and the study discussed above suggesting that surface levels of glutamate receptors may be altered, we also asked whether APOE genotype also affected normal excitatory neurotransmission at the synapse by analyzing the elements of the glutamate-glutamine cycle in the brain (Chapter V). We examined the production and metabolism of glutamate (GLU) and glutamine (GLN) by using high frequency $^{13}\text{C} / ^{1}\text{H}$ nuclear magnetic resonance (NMR) and found that incorporation of $^{13}\text{C}$ label from glucose into C4 and/or C3 isotopomers of glutamate was decreased in APOE-ε4 TR mice. However, APOE-ε4 TR mice had higher levels of brain GLN. Moreover, we found that APOE-ε4 TR mice had lower levels of glutaminase, the enzyme
responsible for converting GLN to GLU, and higher levels of the VGLUT1 transporter, a transporter involved in GLU packaging. Taken together, these data suggest that APOE genotype affects presynaptic terminal composition and impacts the normal GLU-GLN cycle.

It is possible, that similar to our discussion about ApoE affecting post-synaptic terminal composition, that these are due to altered APOE signaling through ApoE receptors. To begin examining these, one would need to better profile which ApoE receptors are pre-synaptic versus post-synaptic. To date, evidence suggests that ApoE receptors are predominantly in the post-synaptic compartment (Chapter IV, (59, 185). However, a more thorough examination is needed as not all ApoE receptors have been characterized at synaptic terminals. Future in vitro studies will address whether apoE receptors are involved in modulating the presynaptic (glutamate synthesis, VGLUT1 levels, glutaminase levels) and postsynaptic (GLU trafficking, dendritic spines) effects of ApoE4, to begin elucidating the mechanism of these isoform specific effects.

It is also possible that ApoE effects at the post-synaptic terminal in turn impact the presynaptic terminal composition. For example, our lab has shown that post-synaptic ApoEr2 can induce pre-synaptic clustering using heterologous co-culture systems (Dumanis, et al 2011, n.b. data not shown in chapter IV). Alternatively, studies have shown that treating rats with NMDA receptor antagonists, such as MK-801, can reduce glutaminase levels (186). Thus, post-synaptic reduction of NMDA receptor surface levels in APOE-ε4 TR mice could potentially explain presynaptic reductions of glutaminase levels.

In our studies, it is not clear which change (i.e., pre or post synaptic alterations) happens first, or if they occur independently of one another. From the time points considered, it seems as if both of these events (altered pre and post synaptic elements) occur at early time-points, giving
no indication that one precedes the other. Perhaps if we examined even earlier time-points, we could determine whether the dendritic spine densities or altered neurotransmitter levels or altered glutaminase and vGLUT1 levels occurred first. Another possibility is to move to an *in vitro* neuronal/glial co-culture system. The glial cultures derived from APOE-ε3 or APOE-ε4 TR mice would be able to generate the apoE particles that would be delivered to the neurons. The neurons could be derived from ApoE deficient mice, to ensure that there is no endogenous neuronal apoE produced. ApoE is rarely synthesized in neurons, but can in moments of severe stress to the cell (187). As the time course for synaptic development in primary neuronal cultures is well defined, this is an ideal system to examine how the different ApoE particles can impact early pre- and post-synaptic events in the maturing neuron.

Interestingly, another study, which also found that ApoE-ε4 TR mice have increased levels of VGLUT1 compared to the other genotypes, was able to modulate VGLUT1 levels with diet, by altering the intake of fish oil (DHA) and cholesterol (138). These studies suggest that diet may interact with ApoE dependent effects. It would be interesting to repeat these studies and examine whether GLN levels or glutaminase can also be modulated by these dietary changes.

**What is the functional consequence of having these biochemical alterations at the synapse?**

These APOE-dependent changes at the synapse suggest that there could be alterations in neuronal signaling. Specifically, if there are reduced dendritic spines, this may indicate a reduction in post-synaptic signaling, which is often measured through long term potentiation (LTP) and mini excitatory post synaptic current (mEPSC) amplitudes. One study, examining
LTP in the dentate gyrus by stimulating the perforant path in 2-4 month old APOE TR hippocampal slices, found that LTP was decreased in APOE-ε4 TR mice compared to APOE-ε3 TR mice (145). While another study, which stimulated the Shaffer collaterals in 3-5 month old APOE TR hippocampal slices, reported that LTP was increased in the CA1 region in the APOE-ε4 TR mice compared to the APOE-ε3 TR mice (36). Due to the complex circuitry of the hippocampus, it is possible that the regional differences result from the dentate gyrus and CA1 differing synaptic connections with various brain regions. For example, the entorhinal cortex inputs into the dentate gyrus which subsequently inputs into CA3 to CA2 to CA1. Therefore, it is possible that CA1 input is compensation for less input into the initial system. No studies have reported changes in mEPSC amplitudes or examined LTP in the cortex, which is where we report differences in dendritic spine density.

Our observed effects for GLU and GLN have been averaged across brain regions, due to the limitations of NMR sensitivity, we were using whole brain extracts enriched with $^{13}$C label to achieve our signal. Future studies will begin to elucidate the brain specific region effects of APOE-ε4 TR compared to the other genotypes. We can enhance our NMR signal by either pooling brain region samples together or by using double labeled $^{13}$C Glucose. We were using $[1^{-13}C]$ glucose, however if we used $[1,3^{13}C]$ glucose, we would increase the amount of pyruvate labeled before it entered the TCA cycle. These studies are worth pursuing, as it would be interesting to determine whether our pre-synaptic effects are also dependent on the brain region examined.

Pre-synaptic signaling is usually measured by examining mEPSC frequency, which is indicative of the quantel content, as well as through paired-pulse facilitation protocols. The
studies on paired-pulse facilitation have not been thoroughly examined but one study reported that APOE-ε4 TR mice in 1 month and 7 month old slices have a decrease in mEPSC frequency in the amygdala (188), suggesting that there may be abnormal pre-synaptic signaling in the amygdala.

Alterations in signaling could also impact the balance between excitatory and inhibitory neurotransmission. A recent study has reported an emergence of a seizure phenotype in a small percentage of aged APOE-ε4 TR mice (41). We have also observed this seizure phenotype in 10% of our colony (data not shown), corroborating these reports, suggesting that there may be alterations in the precise excitatory and inhibitory signaling balance. The exact cause of these seizures are unknown, and the seizures seem to follow a large 24-48 hour refractory period before the animal can be observed to outwardly seize again. Due to the difficulty in determining the source of the seizures and the small percentage of animals which seize, another outcome measure is preferable. It would be interesting to look at the cortical EEG signaling in our APOE-ε4 TR colony, to determine whether those not having seizures still have abnormal cortical signaling. Furthermore, human-ε4 carriers have altered cortical EEG signaling following hyperventilation, suggesting that these potential abnormalities may translate to the human population (146).

Because we had observed increased levels of VGLUT1, we wondered whether this was associated with an increase in overall pre-synaptic vesicles or whether this meant that more VGLUT1 was on each vesicle. To assess this, we used transmission electron microscopy, and determined the density of synaptic vesicles at excitatory pre-synaptic terminals (Chapter VI). We found that the overall density of pre-synaptic vesicles was unaltered, but the distribution profiles
between the APOE genotypes were different. Specifically, the APOE-ε3 TR mice seem to have two distinct types of pre-synaptic terminals, one with few synaptic vesicles (density ranging from .00025 to .00045 vesicles/nm²) and another population of greater synaptic vesicles (density ranging from .0005 to .0075 vesicles/nm²). The APOE-ε4 mice did not display this phenotypic distribution, but had more of a Gaussian distribution. For example, 30% of the ε3 vesicles were in the .005-.0075 range, while only 11% of the ε4 vesicles were. This difference in distribution could impact synaptic vesicle release.

Given the difficulty of quantifying differences in presynaptic vesicles in EM images, it may be necessary to use other techniques. To measure synaptic release, we could move to an in vitro system to measure the exocytosis rates of vesicles (171). Briefly, primary neurons derived from APOE TR mice can be transfected with synaptopHluorin (SPH), which is a SNARE protein fused to a pH sensitive GFP. SNARE proteins are involved in the docking of vesicles to the membrane allowing for neurotransmitter release (171). As soon as the synaptic vesicle fuses to the membrane, the sudden rise in pH will potentiate the fluorescence of SPH, which can be visualized via a live cell imaging microscope. We could therefore measure the difference in fluorescence as well as the rate in which the fluorescence changes following various experimental set-ups can utilize different depolarization protocols (i.e., 50 mM KCl) to elicit vesicle release.

What are the functional consequences of altered GLN levels?
The most striking phenotype that we have observed at the synapse was the elevated levels of GLN, with APOE-ε4 TR mice having over a 2 fold increase in GLN levels compared to the other APOE genotypes. These elevated levels could be due to GLN not being converted to GLU in the presynaptic terminal, or it could be due to GLN levels being trapped in astrocytes and not being shuttled over to neurons for breakdown to GLU. Although we examined many elements of the GLU-GLN cycle, we did not examine the expression of Na+ dependent system-N or Na+ independent system L transporters that are involved in astrocyte to neuron GLN delivery (189). As astrocytes are a key component of brain ApoE biology, these transporters should not be ignored as another potential mechanism by which GLN levels are altered.

Although GLN is traditionally classified as a non-essential amino acid that can cross the blood brain barrier (150-151), because many cells require GLN for survival, it has recently been re-classified as a “conditionally essential” amino acid (160) for the body. GLN is typically considered neuro-protective. For example, oxidation of GLN, via glutaminolysis, can be used as an alternative energy source to drive mitochondrial ATP (158-159). However, too much GLN is associated with neurotoxicity and correlates to levels of brain swelling in patients with hepatic encephalopathy (HE). Moreover, the brain swelling in HE patients is treated with methionine sulfoximine (MSO), a glutamine synthetase inhibitor, which inhibits GLN synthesis (161). *In vitro* studies, in glial/neuronal mixed cultures, showed that high levels of GLN treatment induced mitochondrial swelling and oxidative stress (162). The mechanism by which GLN causes this toxicity has been labeled the “Trojan Horse” hypothesis. This hypothesis postulates that because GLN is easily transported into mitochondria where it can be hydrolyzed to ammonia and glutamate via the glutaminase enzyme. Therefore, ammonia which normally wouldn’t diffuse
into the mitochondria can now reside there and induce toxicity (163). Indeed, the GLN induced mitochondrial stress in vitro can be alleviated by inhibiting glutaminase (162). The generation of high levels of ammonia inside the mitochondria is believed to cause mitochondrial permeability transition, mitochondrial swelling and oxidative damage (164-165).

To test whether the high levels of GLN observed in the APOE-ε4 TR mice resulted in mitochondrial swelling, we used transmission electron microscopy to examine the size and phenotype of mitochondria. We found the opposite of what we hypothesized. Specifically, we found that there was a 10% decrease in mitochondrial diameter width in pre-synaptic terminals of APOE-ε4 TR mice compared to APOE-ε3 TR mice (Chapter V). Mitochondrial surface area is believed to impact calcium and ATP buffering (166-167). The 10% decrease in the cross-sectional mitochondrial surface area observed in APOE-ε4 TR mice could imply reduced mitochondrial efficiency. Future studies, with isolation of mitochondria from synapses of APOE-ε3 and APOE-ε4 TR mice could test for ATPase activity to determine whether this is the case. Interestingly, two recent studies support the hypothesis that APOE-ε4 genotype impacts mitochondria efficiency. A recent proteomics study reported that APOE-ε4 TR mice have reduced levels of Complex I subunits compared to APOE-ε3 TR mice (168). Another study examined postmortem tissue and reported that young APOE-ε4 human carriers, without any amyloid pathology, had lower levels of cytochrome oxidase (the Complex IV component of the electron transport chain) compared to non-APOE-ε4 carriers (169).

Validating GLN as a biomarker for AD
To address whether increased GLN levels in the APOE-ε4 TR mice translated to the human populations, we analyzed CSF samples from cognitively normal individuals, drawn from the three most common APOE genotypes: APOE ε2/ε3, APOE ε3/ε3, and APOE ε3/ε4. We found that APOE ε3/ε4 individuals had significantly increased levels of GLN compared to ε2/ε3 individuals, and a trend toward an increase compared to ε3/ε3 individuals. As discussed in Chapter VII, our hypothesis is that those ε3/ε3 individuals who had higher levels of GLN would be at increased risk of getting the disease. To extend these studies, recently we received samples from Washington University’s Alzheimer’s Disease Research Center that will allow us to increase our sample size to 20 individuals of each of the common genotypes (εX/ε3, previously we had examined 6-7 samples each), and are also comparing these values to 5 individuals with the rare APOE–ε4/ε4 genotype. This increase would allow us to determine whether APOE–ε3/ε4 individuals have significantly higher levels of glutamine in their CSF, and whether there is increased variance in the measures of this metabolite in individuals at higher risk of AD.

Moreover, we would be able to ask whether individuals homozygous for ε4, had greater levels of GLN, indicating the greater risk of getting AD.

If we do find a significant correlation between GLN levels and APOE status, we can then ask at what age the GLN differences occur. For example, a recent study provides evidence that AD related changes begin 2 decades before actual diagnosis (7). Thus, some of the CSF changes in our 50-60 year cohort may be due to the disease process already beginning, despite the individuals appearing cognitively normal. By examining younger ages (for example, controls in
their 20’s), we could better delineate APOE effects from AD related changes. Moreover, we could also follow these donors as they age, to determine whether those with higher levels of GLN do indeed get diagnosed with AD later on in life. If this is indeed the case, GLN will be a novel biomarker that could be used as an outcome measure in preventative and clinical trials.

APOE-\(\varepsilon4\) increases the risk for AD, but an APOE- \(\varepsilon4\) carrier isn’t guaranteed to get AD. If GLN is indeed a marker for increased risk for AD, than we would expect that there are some APOE-\(\varepsilon4\) carriers, who do not have high levels of GLN, and may be less likely to be diagnosed with AD compared to APOE-\(\varepsilon4\) carriers who have high levels of GLN. In our own population, we observed 2 APOE-\(\varepsilon4\) carriers who had lower levels of GLN. We would predict that these two APOE-\(\varepsilon4\) carriers would not go on to get AD. If these predictions hold true, the question remains about whether GLN is just a biomarker or whether it is involved in the disease. If it is involved in the disease process, than treatments to reduce GLN levels could be tried in preventative trials. For example, one might start giving MSO treatment, which is already clinically approved for HE, to those with increased levels of GLN as a preventative measure. However, it isn’t clear whether GLN is a causative factor to the differences observed at the synapse or rather it is an indirect measure of an underlying bigger problem. Even if it isn’t the cause behind the increased risk, this measure would allow us to better identify the population that is at increased risk for AD, and to enroll these particular patients into clinical trials similar to the ones going on right now for familial AD patients currently asymptomatic for the disease.

Epidemiological studies highlight how AD can be a multi-factoral disease. There are various factors that can impact one’s risk for AD. For example, increased blood pressure (190), increased levels of cholesterol (191), and hyperinsulemia (192) all enhance one’s risk for AD. In
contrast, statins (193), light to moderate alcohol consumption (194), exercise (195), and non-steroid anti-inflammatories (NSAIDS) (196) decrease one’s risk for the disease. Intervention trials implemented in elderly populations aimed at lowering risk factors (by lowering blood pressure or cholesterol) and enhancing preventative factors such as increasing NSAID usage have been unsuccessful, and in some cases, have even increased the prevalence of AD among the cohorts studied (197-200). These failed trials highlight two important issues: (1) what is the appropriate time window of interventions (are we intervening when it is too late in the biological cascade?), and (2) what are the appropriate outcomes to measure? As outcome measures, clinicians are examining clinical dementia ratings, and $A\beta$ and Tau levels. However, these markers are also used to define the disease, and would therefore be ineffective if we were to examine younger healthier populations. Interestingly, APOE can interact with some of these risk factors. For example, exercise and NSAID usage seems to have greater protective effects against AD in the APOE-ε4 carrier population compared to non-APOE-ε4 carriers (201-202). Therefore understanding what makes an APOE-ε4 carrier’s brain more susceptible to AD compared to a non-APOE-ε4 carrier, may be instrumental in understanding the other underlying risk factors for the disease. Moreover, these characterizations will hopefully better inform us on the appropriate time window for targeted intervention against AD.

Conclusions

Our hypothesis was that APOE affects normal brain function independent of AD pathology. To test this, we used APOE TR mice, which have human APOE alleles knocked in to
replace the murine APOE, and do not display any AD pathological changes (Sullivan et al, 1997). We found that despite the lack of AD pathology, APOE−ε4 TR mice had alterations at the synapse. Specifically, APOE-ε4 TR mice have fewer dendritic spines at the post-synaptic terminal. We found that the apoE receptor, ApoEr2, is involved in dendritic spine formation, and may be the mechanism by which apoE isoforms differentially affect spine morphology. Pre-synaptically, APOE-ε4 TR mice have reduced levels of glutaminase, increased levels of VGLUT1 and increased levels of GLN. These changes suggest that ApoE4 may influence synaptic signaling, and future studies will test this hypothesis. Moreover, these studies suggest that GLN may be a novel biomarker used to assess AD patients in their pre-clinical phases. Verification of these studies would allow clinicians to use GLN as a therapeutic measure in preventative AD trials.
Appendix A: Glutamatergic Receptor Expression Levels in APOE Targeted Replacement mice

Our ultimate goal is to find viable biomarkers that can be used to better assess therapeutic outcome in Alzheimer’s disease (AD). We are proposing to do this by identifying differences in an at-risk AD group (APOE-ε4 carriers) versus a not-at-risk AD group (APOE-ε4 negative carriers).

In chapter III, we observed that APOE-ε4 TR mice had a decrease in dendritic spine density compared to the other APOE genotypes. This observation was confirmed in three independent cohorts at multiple ages, with the earliest being 4 weeks of age. Dendritic spine counting is a time consuming process, with the golgi staining taking over 2 weeks before one can even begin the sectioning and imaging of the regions of interest. We wanted to determine whether a quicker biochemical assay could be used to assess the differences in the APOE TR mice for potential future pre-clinical animal trials. Because dendritic spines are sites of excitatory postsynaptic transmission, we asked whether the reduction in dendritic spine correlated to altered glutamate subunit composition in the APOE-ε4 TR mice compared to the other genotypes. To address this question, we homogenized 3-4 month old brains of APOE TR mice (n=4/genotype) in RIPA buffer and probed for AMPA and NMDA receptor subunits levels via western blot.

Results:

At 3-4 months of age, we did not observe any differences in the AMPA receptor subunits, GluA1 and GluA2, or in the NMDA receptor subunits, NR1 and NR2A. We did however,
observe, over a 2 fold increase in NR2B receptor subunit expression in APOE-e4 TR mice compared to the other genotypes (Fig A.1A,B). Since AD is an age dependent process, we wanted to assess whether this difference increased with age. Therefore, we homogenized 6 month old APOE TR mice (n=4/genotype) in RIPA buffer and immunoblotted for the AMPA and NMDA receptor subunits as before. As we saw in the 3-4 month cohort, there were no differences in GluA1, GluA2, NR1 and NR2A subunit expression (data not shown). Interestingly, the NR2B receptor subunit levels were also at similar levels between the genotypes (Fig A.1C), in contrast to what was seen earlier.
Figure A.1 NMDA and AMPA receptor subunit expression levels in APOE TR mice. The cortex of APOE-ε2, APOE-ε3 and APOE-ε4 TR mice were homogenized in RIPA buffer and immunoblotted for NMDA and AMPA receptor subunits. (A) Representative images of NMDA and AMPA receptor subunit expression at 3-4 months is shown with β-actin as a loading control. (B) Quantification of NR2B receptor subunit expression at 3-4 months of age. Data is expressed as % of APOE-ε3 levels. Error bars are S.E.M. *, p<0.05. (C) Representative image of NR2B levels at 6 months of age demonstrating that NR2B levels are similar between genotypes with β-actin as a loading control. n=4/genotype/time-point.
Discussion:

In early development, glutamatergic signaling is predominantly through the NMDA receptors (203). Functional NMDA receptors are heteromeric complexes, composed on the NR1 subunit and one or more NR2A-D subunits. The NR1/NR2B composition is highest in early development, and is associated with longer excitatory post synaptic currents (EPSCs). NR1/NR2A receptors have faster decay kinetics resulting in shorter EPSC currents (204). Our finding that NR2B levels were over 2 fold higher in the APOE-ε4 mouse brain at 3-4 months of age compared to the other APOE genotypes but not at 6 months suggests that APOE-ε4 TR mice may have altered glutamatergic receptor signaling early in development.

The NMDA receptor subunit switch from NR2B to NR2A has been documented to occur at around 3 weeks in the normal rodent brain (205). Therefore, it would be interesting to determine whether the increase in NR2B levels in the APOE-ε4 TR mice is due to a delay in the developmental switch or an overall increase in NR2B receptor subunit expression at birth. For example, it is possible that at P0, all APOE genotypes express similar amounts of NR2B receptor subunit, but that the switch to NR2A receptor subunits occurs later for the APOE-ε4 TR mice which is why we observe the altered levels. There switch from the NR1/NR2B to NR1/NR2A receptor subunit composition has been correlated with the close of the critical period in development (203, 206). Therefore, if there was an increased critical period window for the APOE-ε4 TR mice this would have implications for the Antagonistic Pleitropy Hypothesis for APOE.
The APOE Antagonistic Pleitropy is an evolutionary biological concept that proposes that APOE genotype impacts cognition and neuronal integrity differently during different life stages (for review see (207)). Although APOE has been thoroughly studied in older populations with the APOE-ε4 allele consistently associated worse cognitive outcome, only in the past decade have researchers begun to examine APOE’s effects in early life. In contrast to studies in elderly APOE carriers, young APOE-ε4 carriers have reported higher IQs, increase verbal fluency and as infants produce higher scores on the 24 month Mental Development Index of the Bayley scale (208-210). In APOE TR mice, no studies to date have been published on behavior in young mice. However, our own lab has found that 3 month old APOE-ε4 TR mice perform worse on the Barnes Maze compared to the other genotypes (Gus Rodriguez, manuscript in preparation) suggesting that APOE-ε4 TR mice do not have beneficial effects on spatial memory at 3 months of age. It is possible that Barnes Maze Task should be assessed even earlier than 3 months to observe potential beneficial effects of APOE-ε4 in mice. However, because NR2B is associated with critical period windows, it might be better to test these mice on sensory deprivation adaptation tasks (ie. whisker trimming and cortical barrel development), as these critical period timing and tasks have been well documented in rodents.

We examined NR2B levels in RIPA extractions, which would not delineate between synaptic and extra synaptic localizations of the NR2B receptor. There is evidence that the localization of the NR2 subunits may have distinct roles in synaptic plasticity, with extrasynaptic NR2 subunits being involved in long term depression compared to those expressed at the synapse being involved in long term potentiation (211-212). Therefore, it would be interesting to do other
extraction procedures, such as synaptosomal fractionations, to determine whether this increase in NR2B is in the post synaptic density or not.

To further examine potential differences in glutamatergice receptor signaling among the APOE-TR mice, one could use electrophysiology. Studies in APOE-TR slices, examining how APOE genotype affects basal synaptic transmission are mixed. One study, which stimulated the Shaffer collaterals in 3-5 month old APOE TR hippocampal slices, reported that NMDA-dependent long term potentiation (LTP) was increased in the CA1 region in the APOE-ε4 TR mice compared to the APOE-ε3 TR mice (36). However, another study, stimulated the perforant path in 2-4 month old APOE TR hippocampal slices and found that NMDA-dependent LTP was decreased in APOE-ε4 TR mice compared to APOE-ε4 TR mice (145). Due to the complex circuitry of the hippocampus, it is possible that the regional differences result from the dentate and CA1 differing synaptic connections with various brain regions. Another study reported that slices from 1 month and 7 month old APOE-ε4 TR mice have a decrease in mini Excitatory Post Synaptic Currents (mEPSC) frequency in the amygdala (188). These studies illustrate that there is abnormal signaling in APOE-ε4 TR mice; however, why the signaling is altered is still unclear. To determine whether altered NR2B receptor subunit composition can explain some of these differences, slices could be bathed in ifenprodil, an NR2B receptor agonist, compared to control solution to assess whether differences in basal synaptic transmission via mEPSC amplitude and frequency, as well as NMDA-dependent LTP are altered due to altered NR2B composition.

**Conclusions:** The surprising finding that NR2B levels are increased at 3 months but not at 6 months in APOE-ε4 TR mice compared to the other genotypes, leads the door open for
many possible areas of exploration in electrophysiology and behavioral studies. These studies can assess whether APOE-ε4 TR mice have enhanced function in early development, which they perform worse on in later adulthood.
Appendix B: Proton Magnetic Resonance Spectroscopy Imaging in APOE Targeted Replacement mice

Proton magnetic resonance spectroscopy (\(^1\)H MRS) is a noninvasive way to measure in vivo neuro-chemical changes. Therefore, \(^1\)H MRS is ideally suited to obtain information about dynamic physiological profiles of metabolites in the brain. Moreover, if biomarkers are found among these brain metabolites, they can be used to better assess preventative therapies in Alzheimer’s disease (AD).

This technique is already being used in the AD clinic, with studies reporting decreases in N-acetylaspartate to creatine (NAA/Cr) and increases of myoinositol to creatine (mIns/Cr) and choline to creatine (Cho/Cr) in mild cognitive impairment patients that progress to AD compared to age-matched cognitively normal controls (213-219). Recently, there have also been reports of decreased Glutamate (Glu) levels in AD patients, although the Glu peak is hard to delineate between the glutamine peak due to spectral resolution issues (213, 220). These potential biomarker changes have been used to interpret what might be going on in the AD brain.

These metabolites could provide insight into the biological processes occurring in the brain. NAA in the CNS is predominantly localized to neurons, and is a therefore used as a marker for neuronal viability. Moreover, it is believed that NAA is produced in the mitochondria, and a correlation has been established between mitochondrial ATP production in neurons and NAA levels (221), implying that it may also be a marker for neuronal mitochondrial function. mIns, however, is predominantly localized in glial cells (222).
Elevated levels of mIns are correlated with glial inflammation, disregulated osmoregulation and proliferation (223). Interestingly, taurine (Tau) is an inhibitory neurotransmitter in the brain, and in rodents is hypothesized to take over mIns’s role in osmoregulation (224).

The majority of Cho resides in choline-bound membrane phospholipids, and therefore Cho decreases are correlated with loss of membrane integrity while elevation of the Cho peak is postulated to be a consequence of increased membrane turnover due to neurodegeneration (225). Although consistent literature has been reported reductions in Cho in stable mild cognitively impaired patients, in AD patients the reports of Cho levels are inconsistent, potentially due to acetylcholine esterase inhibitors prescribed for AD patients, which could affect the choline that is produced by acetylcholine esterase (226).

Creatine (Cr) is utilized as an energy reservoir, involved with ATP buffering (227). Specifically, creatine kinase converts Cr to Cr phosphate, a high energy phosphate, allowing cells to store adenosine triphosphate (ATP). A majority of studies report that total Cr levels are unaffected in AD, and therefore have used it as an internal reference compared to the other metabolites (228).

Our ultimate goal is to find viable biomarkers of APOE pathogenesis that increase one’s risk of getting AD. To beginning addressing this question, we used $^1$H-MRS in 20 month old male APOE-ε4 and APOE-ε3 TR mice (n=6/genotype), to address whether we could observe any biochemical differences in NAA, mIns, Cr, and Cho peaks, as these are the differences that have been reported in the literature. Due to our findings that the glutamate-glutamine cycle is
disrupted (see Chapter V), we were also interested in examining the glutamate/glutamine peak among the APOE genotypes.

**Results**

Using single-voxel proton Magnetic Resonance Spectroscopy, 20 month old male APOE-ε3 and APOE-ε4 TR mice were analyzed in a 7 Tesla Bruker horizontal bore Magnetic Resonance Imager. Based on Magnetic Resonance brain anatomical images, a voxel was positioned over the hippocampus with the resulting spectral profile (Fig B.1A). From the resulting biochemical profile, one can distinguish the Cr/pCr (total Cr levels), Cho (Choline), NAA/NAAG, glutamate-glutamine (GLU/GLN) and taurine (Tau) levels.

Using creatine as an internal reference, all of the areas of the peaks were calculated and divided by the creatine area. Between the APOE genotypes there were no differences in the chemical profiles (Fig B.1B). To ensure that creatine levels were not different among the genotypes, we used a second reference, and set the area under the Cho peak to one for all of the resulting spectral profiles, including the total Cr area (Fig B.1C). With these analyses, there were also no differences between the APOE genotypes.
Figure B.1 Resulting $^1$H MRS Spectral Profiles in ages APOE TR males. Six 20 month old APOE-ε3 and APOE-ε4 TR males were placed in a 7 Tesla Bruker horizontal bore Magnetic Resonance Imager. (A) A representative image of an APOE-ε4 mouse brain showing where the voxel was positioned over the hippocampus (left inset) and the resulting spectral profile was produced. (B) Using Cr/pCr as an internal standard, the area under the curves were calculated and divided by Cr/pCr. Error Bars are S.E.M. (C) To determine whether Cr/pCr levels were not different between the two groups, the Cho area was set to 1 to normalize the values for all of the calculations, and the Cr/pCr (total Cr) area was calculated. Error bars are S.E.M.
**Discussion:**

These preliminary studies indicate that $^1$H-MRS cannot be used to distinguish between two different biochemical profiles in APOE-ε3 and APOE-ε4 TR mice. Our voxel was positioned in the hippocampus and it is possible that there were differences in other brain regions. For example, although we observed APOE dependent effects on dendritic spine density in cortical layers II/III, we did not observe any differences in dendritic spine density in the dentate gyrus (Chapter III). Moreover, this preliminary study used males, but it is possible that females may have altered biochemical profiles even if the males do not display different spectral profiles. For example, one study reported that female APOE-ε4 TR mice displayed more severe spatial memory behavioral deficits compared to males and at earlier ages (39). Overall, however, these studies suggest that in adult males, $^1$H-MRS, at its current sensitivity, could not identify any potential differences in metabolite profiles in the APOE TR mice.
Appendix C: Publications While Completing Thesis Work at Georgetown

**Research Papers**


*Featured Article in Journal Club Section of The Journal of Neuroscience, March 31, 2010*


**Dumanis SB**, Chamberlain KA, Sohn YJ, Young JL, Suzuki T, Mathews PM, Pak DTS, Rebeck GW, Suh YH, Park HS, Hoe HS. (2012) FE65 as a link between VLDLR and APP to regulate their trafficking and processing. Molecular Neurodegeneration. 7(1). PMCID: PMC3379943


*Featured Article in the Editorial Commentary in the 2013 Journal of Neurochemistry 124(1)*

Washington, PM, **Dumanis SB**, Zapple D, Burns M. (Submitted, under review). Clearance of brain injury-induced amyloid-beta is impaired in APOE4 genotype mice.
Educational Papers


*Abstract from SFN regarding this paper was featured in Science’s Career Blog 11/15/2011
Bibliography:


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therapeutics rapidly clear beta-amyloid and reverse deficits in AD mouse models. *Science* 335: 1503-6


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206. Erisir A, Harris JL. 2003. Decline of the critical period of visual plasticity is concurrent with the reduction of NR2B subunit of the synaptic NMDA receptor in layer 4. *J Neurosci* 23: 5208-18


cognitive impairment (aMCI): a three-year follow-up study. Arch Gerontol Geriatr 54: 192-6


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