HUMAN APOE4 AFFECTS MICROGLIAL REACTIVITY AND SPATIAL COGNITION IN A MOUSE MODEL OF ALZHEIMER’S DISEASE RISK

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By

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HUMAN APOE4 AFFECTS MICROGLIAL REACTIVITY AND SPATIAL COGNITION IN A MOUSE MODEL OF ALZHEIMER’S DISEASE RISK

Gustavo Armando Rodriguez, B.A.

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ABSTRACT

Alzheimer’s disease (AD) is a progressive age-related neurodegenerative disorder that results in declarative memory deficits. Apolipoprotein e4 (APOE-ε4) is the strongest genetic risk factor for developing AD, and influences extracellular Aβ plaque deposition and neuroinflammation in humans and in mouse models. APOE-ε4 also influences neuronal morphology and cognitive performance in humans, independent of AD pathology. In this series of experiments, we examined the effects of APOE genotype on AD-related neuropathology and in normal brain function of mice to better understand its role as a potent risk-factor for developing AD.

We analyzed Aβ plaque profiles and Aβ-induced glial activation in the brains of 6-month old EFAD transgenic mice (E2FAD, E3FAD, and E4FAD). Characterization of Aβ plaques revealed larger and more intensely stained plaques in E4FAD mice relative to E3FADs, and increased numbers of compact plaques in the subiculum. Reactive microglia and astrocytes were prominent in EFAD brains. Morphometric analyses revealed greater dystrophy, increased fluorescence intensity, and a higher density of reactive microglia surrounding cortical plaques in E4FAD mice than in E3FADs. Cortical levels of Interleukin1-β (IL-1β) were nearly two-fold greater in E4FAD mice relative to E3FADs.
To investigate the impact of APOE genotype on cognitive performance in mice, we trained 3-month old APOE Targeted Replacement mice (APOE2, APOE3, and APOE4) in a spatial learning and memory task (Barnes maze). APOE4 mice exhibited impaired spatial learning and memory in the maze compared to APOE3 mice. Deficits in spatial learning were detected in a second cohort of 18-month old APOE4 mice. When we examined the dendritic morphology of neurons in the medial entorhinal cortex (MEC), we found significantly shorter dendrites and lower spine densities in basal shaft dendrites of APOE4 mice relative to APOE3, consistent with the deficits found in our young APOE4 mice. We also provide evidence to suggest that Liver X Receptor (LXR) activation – which increases APOE – suppresses overall spiking activity and specifically burst counts in mature hippocampal networks recorded in a multielectrode array.

These data suggest that APOE genotype modulates Aβ-induced neuroinflammatory responses in AD progression, and support a role for APOE in dendritic morphology and normal brain function.
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<table>
<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>A(\beta)</td>
<td>Amyloid beta</td>
</tr>
<tr>
<td>ABCA1</td>
<td>ATP-binding cassette transporter A1</td>
</tr>
<tr>
<td>ABCG1</td>
<td>ATP-binding cassette transporter G1</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AMPA</td>
<td>(\alpha)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Apoe</td>
<td>Apolipoprotein E (mouse gene)</td>
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<tr>
<td>APOE</td>
<td>Apolipoprotein E (human gene)</td>
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<tr>
<td>ApoE</td>
<td>Apolipoprotein E (protein)</td>
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<tr>
<td>ApoER2</td>
<td>Apolipoprotein E receptor 2</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cLTP</td>
<td>Chemical long-term potentiation</td>
</tr>
<tr>
<td>CA1</td>
<td>Cornu Ammonis 1 (region of the hippocampus)</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CISH</td>
<td>Chromogenic in situ hybridization</td>
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<tr>
<td>CLU</td>
<td>Clusterin (human gene)</td>
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<tr>
<td>CM</td>
<td>Conditioned media</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine hydrochloride</td>
</tr>
<tr>
<td>Dab1</td>
<td>Disabled 1</td>
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<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole (fluorescent stain; nuclear marker)</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>DIV</td>
<td>Days in vitro</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EC</td>
<td>Entorhinal cortex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
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<tr>
<td>EFAD</td>
<td>APOE x 5xFAD mouse</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>EOAD</td>
<td>Early-onset Alzheimer’s disease</td>
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<tr>
<td>EPSP</td>
<td>Excitatory post-synaptic potential</td>
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<tr>
<td>FAD</td>
<td>Familial Alzheimer’s disease</td>
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<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein (1&lt;sup&gt;o&lt;/sup&gt; antibody; activated astrocytes)</td>
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<tr>
<td>GWAS</td>
<td>Genome wide association study</td>
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<tr>
<td>HD</td>
<td>Hidden platform training day</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HPWM</td>
<td>Hidden platform water maze</td>
</tr>
<tr>
<td>Iba1</td>
<td>Ionized calcium-binding adapter molecule 1 (1&lt;sup&gt;o&lt;/sup&gt; antibody; activated microglia)</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin 1 beta (cytokine)</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6 (cytokine)</td>
</tr>
<tr>
<td>ITI</td>
<td>Inter-trial interval</td>
</tr>
<tr>
<td>ISI</td>
<td>Inter-spike interval</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRP1</td>
<td>Low density lipoprotein receptor-related protein 1</td>
</tr>
<tr>
<td>LTM</td>
<td>Long-term memory</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
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<tr>
<td>LXR</td>
<td>Liver X receptor</td>
</tr>
<tr>
<td>MEA</td>
<td>Multielectrode array</td>
</tr>
<tr>
<td>MEC</td>
<td>Medial entorhinal cortex</td>
</tr>
<tr>
<td>MOAB2</td>
<td>Monoclonal antibody 2 (1&lt;sup&gt;o&lt;/sup&gt; antibody; Aβ&lt;sub&gt;1-42&lt;/sub&gt;)</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>NFT</td>
<td>Neurofibrillary tangles</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>oAβ</td>
<td>Oligomeric Aβ</td>
</tr>
<tr>
<td>OFT</td>
<td>Open field test</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PICALM</td>
<td>Phosphatidylinositol binding clathrin assembly protein (gene)</td>
</tr>
<tr>
<td>PSEN1</td>
<td>Presenilin 1</td>
</tr>
<tr>
<td>PSEN2</td>
<td>Presenilin 2</td>
</tr>
<tr>
<td>RAP</td>
<td>Receptor-associated protein</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>TBI</td>
<td>Traumatic brain injury</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline w/ Triton X-100</td>
</tr>
<tr>
<td>TD</td>
<td>Training day</td>
</tr>
<tr>
<td>TH</td>
<td>Target hole</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TREM2</td>
<td>Triggering receptor on myeloid cells 2</td>
</tr>
<tr>
<td>VLDLR</td>
<td>Very low density lipoprotein receptor</td>
</tr>
<tr>
<td>VT</td>
<td>Visible platform trial</td>
</tr>
<tr>
<td>WAAS</td>
<td>Weak aversive auditory stimulus</td>
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CHAPTER I

BACKGROUND & INTRODUCTION

A. Alzheimer’s disease

Alzheimer’s disease (AD) is a progressive age-related neurodegenerative disorder that results in declarative memory deficits and an impaired ability to function in daily life. Initial AD symptoms include difficulty remembering recent conversations, names or events, and gradually worsen to include impaired long-term memory (LTM). In addition, individuals suffering with AD experience difficulty completing familiar tasks and require assistance with basic activities of daily living. In the AD brain, hallmark pathological insults include brain atrophy due to synaptic and neuronal cell loss, extracellular plaques made-up of amyloid beta (Aβ), intracellular hyper-phosphorylated tau tangles, and glial cell activation that leads to neuroinflammation.

There are two major forms of AD: familial and sporadic AD. Familial AD (FAD) is an early-onset form of the disease (<60 years of age) that is caused by dominantly inherited genetic mutations in genes that code for the amyloid precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2). Mutations in these deterministic genes lead to the generation of Aβ plaques in the brain by the abnormal production or aggregation of Aβ peptides. FAD is very rare, affecting an estimated 200,000 individuals in the US (~1% of AD cases) (Thies et al., 2013). The overwhelming majority of AD cases (~99%) are those that occur sporadically in individuals later in life (>65 years of age); these cases are known as late-onset AD (LOAD). LOAD is not caused by mutations in deterministic genes, but rather is likely the result of a combination of genetic, environmental, and lifestyle factors that influence a person’s risk for developing the disease (see Risk Factors for LOAD, pg. 6).
Epidemiology of AD

AD accounts for 60 – 80% of dementia cases in the United States (US) and affects an estimated 5 million Americans over the age of 65 (Thies et al., 2013). This number is projected to increase to 7 million by the year 2025, which represents a 40% increase in AD cases in only an eleven-year span (Hebert et al., 2013). AD thus has a major impact on individual states’ health care systems and has an economic impact on the country as a whole. In 2013, an estimated 17.7 billion hours of unpaid care - valued at $220.2 billion - was provided to AD patients (Hurd et al., 2013). Family members mostly shoulder this burden, with approximately 55% of primary caregivers taking care of their parents (Fisher et al., 2011). According to the National Center for Health Statistics of the Centers for Disease Control and Prevention (CDC), AD is officially the 6th leading cause of death in the US and the 5th leading cause of death in individuals aged 65 and older. AD-associated mortality in this country is higher than mortality from prostate cancer and breast cancer combined. These statistics highlight an alarming trend for AD prevalence and reveal an underappreciated fact about the disease; AD not only debilitates it victims’ cognitive abilities, but it kills them. An estimated 700,000 individuals will die from AD in 2014 (Weuve et al., 2014), and an increase in AD prevalence within the rapidly growing aging population is expected to increase AD-associated mortality rates.

AD has a particularly strong effect in women, both as patients and as caregivers. Approximately two-thirds of the US AD population are women (~3.2 million), which is not completely due to women having longer average lifespans than men (2014 AD facts and figures). Genetic and physiological factors may play a role in the increased prevalence of AD in women.
APOE-ε4, the strongest genetic risk factor for LOAD, has an enhanced effect on AD risk in women (Ungar et al., 2014). With regards to caregiving, it is estimated that 60 – 70% of informal AD caregivers are women (2014 AD facts and figures). This predominance of female caregivers is likely due to a number of factors, including attitudes on traditional gender roles and whether the women perceive that they have a choice in the matter. More research is needed to understand the complex connection between women and AD.

A Glimpse into the AD Brain

The following two paragraphs are excerpts from an English translation of Alois Alzheimer’s original 1907 publication titled, “Über eine eigenartige Erkankung der Hirnrinde” (English: On an Unusual Illness of the Cerebral Cortex) (Alzheimer et al., 1995).

“The post-mortem showed an evenly atrophic brain without macroscopic focal degeneration. The larger vascular tissue shows arteriosclerotic change. Specimens which were prepared according to Bielschowsky’s silver method show very striking changes of the neurofibrils. Inside of a cell which appears to be quite normal, one or several fibrils can be distinguished by their unique thickness and capacity for impregnation. Further examination shows many fibrils located next to each other which have been changed in the same way. Next, combined in thick bundles, they appear one by one at the surface of the cell. Finally, the nucleus and the cell itself disintegrate and only a tangle of fibrils indicates the place where a neuron was previously located.”

“Distributed all over the cortex, but especially numerous in the upper layers, there are minute miliary foci which are caused by the deposition of a special substance in the cortex. This substance can be observed without dye, but it is very refractory to dyeing. The glia have developed numerous fibers, moreover, many glial cells show adipose saccules. There is a no infiltration of the vessels, however, a growth appears on the endothelia, in some places also a proliferation of vessels. Considering everything, it seems we are dealing here with a special illness.”

These excerpts from Alzheimer’s report represent the first published record of histological silver staining in the AD brain. They provide an impressive, detailed account of
several hallmark AD neuropathologies, which include: extracellular plaques, intracellular tangles, glial activation, and overall gray matter atrophy.

*Aβ and the Amyloid Cascade Hypothesis*

The “special substance” at the core of Alzheimer’s extracellular plaques is Aβ, a peptide of 36-43 amino acids derived from the sequential cleavage of APP by β- and γ-secretases (Hardy and Selkoe, 2002). Cleavage within the transmembrane region of APP generates a number of Aβ species, including Aβ1-40 and Aβ1-42, which undergo post-secretory aggregation and form insoluble deposits in the brain. Aβ peptides can exist in several stages of aggregation, including soluble monomers and dimers, or as misfolded oligomeric “seeds” that induce further misfolding of Aβ peptides. Although Aβ1-40 is the most common form of the peptide, Aβ1-42 is more fibrillogenic and is associated with increased neurotoxicity (Dahlgren et al., 2002; Jan et al., 2008; Jarrett et al., 1993).

The scientific theory that amyloid “aggregate stress” is the initiating factor in a sequence of events leading to AD dementia is called the *Amyloid Cascade Hypothesis* (Hardy and Selkoe, 2002; Karran et al., 2011). This hypothesis is strongly supported by the identification of the deterministic mutations in genes associated with Aβ generation and formation (see above). In this theory, the aggregation and/or deposition of Aβ leads to the formation of insoluble neurofibrillary tangles (NFT) in neurons, which contributes to neuronal dysfunction, cell death, and ultimately to cognitive impairment. Tau pathology first begins in the transentorhinal region of the temporal lobe before spreading to the entorhinal cortex (EC), the hippocampus, and then to the neocortex (Braak and Braak, 1995). NFT accumulation in the superior temporal sulcus of
AD brains is inversely related to the total number of neurons in the region (Gomez-Isla et al., 1997). NFTs are also closely associated with the clinical symptoms of dementia (Arriagada et al., 1992; Giannakopoulos et al., 2003). These findings establish a link between NFT progression, neuronal loss, and cognitive impairment in AD. Importantly, the progression and distribution of NFTs in the brain is independent from that of Aβ plaques, which follows its own progression and distribution in the brain. Unlike with NFTs, no definitive correlation between Aβ plaques and neuronal cell loss or dementia has been demonstrated (Gomez-Isla et al., 1997; Karran et al., 2011). This lack of correlation would suggest that overall Aβ plaque burden may not be a reliable marker of AD progression or disease severity.

In his seminal 1907 paper, Alois Alzheimer also reported that glial cells “developed numerous fibers”, and that a number of them exhibited “adipose saccules”. These morphological descriptors of glial cell activation likely reflect a chronic state of neuroinflammation in which two main types of glial cells, astrocytes and microglia, contribute to AD pathogenesis. The amyloid cascade hypothesis would suggest that Aβ drives tau pathology and neuroinflammation in AD, with both NFTs and neuroinflammation contributing in some manner to synaptic loss and neuronal dysfunction and death. Astrocytes provide many specialized, supportive CNS functions under normal conditions. They help maintain the blood-brain barrier by providing biochemical support to endothelial cells and help regulate extracellular ion concentration at synapses. In the presence of Aβ deposits, astrocytes become reactive, changing their morphology and secreting a host of immunoregulatory molecules (Meraz-Rios et al., 2013). Microglia are the resident macrophages in the CNS and are responsible for several functions of the innate immune response (Prokop et al., 2013; Tambuyzer et al., 2009). Notably, microglia survey the brain parenchyma
for foreign substances and cellular debris, shifting their activation states to phagocytose the material and clear the microenvironment. Reactive microglia in AD brains migrate to and cluster in and around Aβ plaques, where they secrete inflammatory molecules and internalize/degrade Aβ (Amor et al., 2010; Itagaki et al., 1989).

*Risk Factors for LOAD*

While aging is the greatest overall risk factor for developing LOAD, several genetic risk factors that augment disease pathogenesis have been identified. These genes code for the proteins apolipoprotein E (*APOE*), clusterin (*CLU*), phosphatidylinositol binding clathrin assembly protein (*PICALM*), and triggering receptor on myeloid cells 2 (*TREM2*) (Coon et al., 2007; Guerreiro et al., 2013; Harold et al., 2009). Interestingly, several lifestyle factors that influence the risk of cardiovascular disease also influence the risk of LOAD; these include diabetes, obesity, and metabolic syndrome (Thies et al., 2013). An additional risk factor for LOAD is moderate to severe traumatic brain injury (TBI), which occurs after a head injury that results in a loss of consciousness or post-traumatic amnesia (Thies et al., 2013). While each of these risk factors appears to play some role in AD etiology, *APOE* genotype is the strongest genetic risk factor associated with LOAD (Coon et al., 2007).
**B. Apolipoprotein E**

Apolipoprotein E (ApoE) is a small (34-kDa) secreted glycoprotein synthesized in the central nervous system (CNS) by glial cells, and a major constituent of high-density lipoprotein (HDL) particles in the brain (Beffert et al., 1998). Its functional role is to shuttle lipoprotein particles through plasma and cerebrospinal fluid, where it can deliver its lipid payload to target cells by binding to members of the low density lipoprotein (LDL) receptor family (Figure 1). Normal apoE function is essential in the maintenance of lipid homeostasis in the brain, and has been studied in a variety of neurobiological functions throughout the lifespan. Within the past twenty years, ApoE has received considerable attention from the scientific community due to its association with late-onset Alzheimer’s disease.

In humans, the *APOE* gene is mapped to chromosome 19 and has three common alleles: *APOE-ε2*, *APOE-ε3*, and *APOE-ε4*. The frequency of the alleles in the US population is as follows: ε2, 5-10%, ε3, 70-78%, and ε4, 14-20% (Roses, 1996). Each allele codes for polymorphic forms of apoE that differ by amino acids at residues 112 and 158 (apoE2, Cys-112 & Cys-158; apoE3, Cys-112 & Arg-158; apoE4, Arg-112 & Arg-158) (Zannis et al., 1982). ApoE is 299 amino acids in length; an important functional region of apoE is the amphipathic lipid-binding site (residues 244-72) in the C-terminal domain, which attracts free lipids in the initial formation of lipoprotein particles (Kanekiyo et al., 2014). The ATP-binding cassette transporters A1 (ABCA1) and G1 (ABCG1) are important membrane-associated proteins involved in this process. Another major functional region of apoE is the receptor-binding site (residues 136-150) in the N-terminal domain, which binds to cell surface endocytic receptors belonging to the LDL receptor family (Kanekiyo et al., 2014) (Figure 1).
Figure 1. ApoE and LDL Receptors. ApoE associates with high-density lipoprotein particles in the CNS, and helps to transport cholesterol and triglycerides through cerebrospinal fluid and plasma to target cells in the brain. ApoE then binds to members of the low density lipoprotein receptor family, which include the LDL receptor, ApoER2, very low density lipoprotein (VLDL) receptor, and low density lipoprotein receptor-related protein 1 (LRP1). Receptor mediated endocytosis of apoE-containing lipoprotein particles allows released cholesterol and triglycerides to serve several useful cellular functions, including maintenance of membrane integrity and synaptogenesis.

*Adapted from (Rebeck et al., 2006); Molecular Neurodegeneration*
Although apoE4 and apoE3 bind to the LDL receptors with similar high affinities relative to apoE2, binding properties are likely receptor-dependent and influenced by lipidadation status of apoE. The single amino acid difference between apoE3 and apoE4 has been proposed to affect interaction of the N- and C-terminal domains of the protein (Kanekiyo et al., 2014; Wilson et al., 1991). This may result in changes to the tertiary structure of apoE, which could underlie the dramatic differences in function among the apoE isoforms (Mahley et al., 2009; Weisgraber et al., 1981).

Genome-wide association studies (GWAS) establish APOE as the most significant pathogenic locus associated with AD (Coon et al., 2007; Harold et al., 2009). AD risk is augmented by ε4 gene dose, as the proportions of individuals heterozygous or homozygous for APOE-ε4 who are affected by AD late in life are approximately 47% and 91%, respectively (Corder et al., 1993; Liu et al., 2013). Compared to non-ε4 carriers, a single copy of the ε4 allele confers an increased AD risk by age 75 years of 2- to 3-fold, while two ε4 alleles increase AD risk by 12-fold (Farrer et al., 1997). APOE-ε4 also decreases the age of AD onset in a gene-dose dependent manner (Gomez-Isla et al., 1996; Roses, 1996). Thus, possession of the APOE-ε4 allele is the strongest genetic risk factor for the development of AD (Corder et al., 1993; Strittmatter et al., 1993).

The Role of APOE in Aβ Pathology

Although primarily associated with lipid and cholesterol homeostasis, apoE also regulates aspects of amyloid pathology and Aβ metabolism in the brain. ApoE exerts isoform-specific effects on Aβ aggregation, metabolism, and plaque load in both human AD patients and in
mouse models of AD (Castellano et al., 2011; Grimmer et al., 2010; Hashimoto et al., 2012; Irizarry et al., 2000; Rebeck et al., 1993; Reiman et al., 2009; Schmechel et al., 1993; Tai et al., 2011; Tiraboschi et al., 2004). Levels of Aβ found in the AD brain are strongly affected by APOE genotype, and likely contribute to its role as a major genetic risk factor for AD (Bales et al., 2009; Drzezga et al., 2009; Kim et al., 2009). For review of the role apoE isoforms play in AD-associated Aβ pathology, see Kim et al (2009) and Kanekiyo et al (2014).

Histological analyses of AD brains show that apoE localizes to cortical Aβ plaques (Namba et al., 1991). This direct association prompted in vitro studies into the binding kinetics of apoE and Aβ, which show that Aβ peptides can bind to both the lipid-binding and receptor-binding regions of apoE (Golabek et al., 1995; Strittmatter et al., 1993; Winkler et al., 1999). However, apoE-Aβ interaction appears to depend on several key properties of apoE (e.g. apoE isoform tested, lipidation status of apoE, cellular compartment generating apoE) and Aβ (e.g. conformational state of peptide), in addition to how the apoE-Aβ complexes are evaluated (Kanekiyo et al., 2014). The general consensus is that apoE4 forms less stable complexes with Aβ than apoE3, which may result in less efficient Aβ clearance and increased aggregation and deposition. Consistent with this hypothesis, APOE4 is associated with less Aβ1-42 uptake into microglial cells (Zhao et al., 2014), and Aβ plaque deposition is greater in amyloid-generating transgenic mice that express human APOE4 than APOE3 (Oakley et al., 2006; Youmans et al., 2012b).

**The Role of APOE in Neuroinflammation**
ApoE is involved in modulating both systemic and CNS inflammatory responses independent of Aβ pathology. *In vitro* studies demonstrate that apoE suppresses the lipopolysaccharide (LPS)-stimulated induction and release of proinflammatory molecules, and it promotes macrophage M2 polarization in cells (alternative activation, anti-inflammatory phenotype) (Baitsch et al., 2011; Laskowitz et al., 1997; Lynch et al., 2001). ApoE also suppresses LPS-induced inflammatory gene expression in mouse models (Lynch et al., 2001). These findings demonstrate a functional role for apoE as an anti-inflammatory mediator of the immune response. Interestingly, apoE appears to exert an isoform-specific influence over this role. Administration of LPS into transgenic APOE4 mice results in exaggerated and prolonged neuroinflammatory and neurotoxic responses, which may be due to less effective anti-inflammatory function relative to apoE3 (Lynch et al., 2003; Ophir et al., 2005; Zhu et al., 2012). This hypothesis is supported by several *in vitro* studies examining the influence of apoE4 on LPS-mediated glial activation (Colton et al., 2004).

While a role for apoE in neuroinflammation has been repeatedly demonstrated, the mechanisms underlying the isoform-dependent differences remains unclear. In addition, APOE4-associated AD risk may in part be driven by dysfunctional neuroinflammation in response to Aβ pathology. Suitable *in vitro* and animal models are required to test the influence of human apoE isoforms on Aβ.

**The EFAD Mice**

The EFAD transgenic mouse model was recently developed to investigate APOE genotype-specific effects on AD pathological changes in the brain (Tai et al., 2013; Tai et al.,
2011; Youmans et al., 2012b). In this model, 5xFAD mice expressing mutant FAD forms of APP and PS1 (Oakley et al., 2006) were crossed to APOE targeted replacement mice expressing each of the human isoforms (Knouff et al., 1999; Sullivan et al., 1997; Sullivan et al., 1998). The resulting EFAD mice (E2FAD, E3FAD and E4FAD) exhibit robust amyloid deposition in the brain, even at young ages (Youmans et al., 2012b). Young (2- to 6-month old) E4FAD mice exhibit accelerated Aβ accumulation in the subiculum and frontal cortex, greater total levels of Aβ1-42 in the hippocampus, and selective increases in soluble Aβ1-42 and oligomeric Aβ (oAβ) compared to E2FAD and E3FAD mice. These mice are thus a tractable model for Aβ pathology in the brain, and are well suited for studies examining the role of apoE isoforms in Aβ aggregation, deposition, and clearance. EFAD transgenic mice are also an attractive model for investigating the synergistic effects of APOE genotype and neuroinflammation in Aβ pathology. Glial-mediated inflammatory responses to Aβ are an important mechanism in AD pathogenesis, yet little is known regarding the role of APOE genotype in this process.

* Aβ-Independent Effects of APOE

In order to fully appreciate how APOE genotype may influence AD risk, isoform-specific differences in basic neurobiological function must also be assessed in the absence of Aβ pathology. ApoE exerts isoform-specific effects on normal CNS structure and function in humans. APOE-ε4 carriers have differences in age-related cortical thickness, cognitive decline, and intrinsic functional brain network activity in the absence of AD pathological changes (Brown et al., 2011; Burggren et al., 2008; Caselli et al., 2009; Green et al., 2014; O'Dwyer et al., 2012; Shaw et al., 2007; Stevens et al., 2014; Verghese et al., 2011). APOE-ε4 carriers also
display deficits in episodic memory and show age-related memory decline earlier in life than in non-carriers (Caselli et al., 1999; Nilsson et al., 2006). These findings suggest that APOE genotype differentially modulates cognitive processes during normal aging, and suggest that it may be possible to detect APOE4-related learning and memory deficits early in life.

**APOE Targeted Replacement Mice**

Significant contributions to our understanding of APOE-associated AD risk stem from the generation and study of humanized APOE targeted replacement mice (referred to as APOE mice in Chapters III-VII) (Knouff et al., 1999; Sullivan et al., 1997; Sullivan et al., 1998). These mice feature a targeted replacement of the murine Apoe gene with either human APOE-ε2, APOE-ε3, or APOE-ε4 alleles. Specifically, targeting constructs containing human APOE coding exons 2–4 were used to introduce the human alleles into the Apoe gene locus in place of murine coding sequences (exons 2–4). The resulting chimeric genes retain all the normal mouse regulatory and flanking sequences in addition to non-coding mouse exon 1 (Sullivan et al., 1997). As one would expect, mRNA expression of human APOE parallels that of wild-type mice, with the highest expression in the following tissues: liver, brain, and spleen. Approximately 70% amino acid sequence homology is shared between human and murine apoE protein, with primary differences located at the N- and C-termini (Weisgraber, 1994). Importantly, the receptor-binding domain is highly conserved between human and mouse species.

The three lines of APOE mice (APOE2, APOE3, and APOE4) are an attractive model for behavioral studies of AD susceptibility, as the contribution of each apoE isoform to a variety of
CNS functions can be tested to determine adverse or protective mechanisms that influence AD risk. In this model, human apoE isoforms are expressed in glial cells at physiological levels and no gross pathological changes in brain organization are evident (Kitamura et al., 2004; Korwek et al., 2009; Sullivan et al., 1997). Several differences based on human APOE genotype have already been observed in these mice. Structurally, APOE4 reduces dendritic complexity and spine density in vivo (Dumanis et al., 2009; Wang et al., 2005). Functionally, APOE4 negatively affects aspects of synaptic plasticity (Chen et al., 2010; Korwek et al., 2009; Trommer et al., 2004). APOE4 mice have alterations in elements of the glutamate–glutamine cycle, and exhibit a seizure phenotype correlated with abnormal cortical EEG activity, indicating a disruption in the balance of excitatory and inhibitory neurotransmission (Dumanis et al., 2013; Hunter et al., 2012; Li et al., 2009). These reports provide compelling evidence for abnormal neuronal function in APOE4 mice that may lead to cognitive impairment.

Raber et al. (2000) describe apoE4-specific cognitive deficits in mice using the Morris water maze, a behavioral task designed to assess spatial learning and memory in rodents (Morris, 1984). Aged (18-month old) transgenic female mice expressing human apoE4 under the neuron-specific enolase promoter performed poorly in both the training trials (learning phase) and probe trial (memory) of the task in the absence of Aβ pathology (Raber et al., 2000). Behavioral studies utilizing human APOE targeted replacement mice have reported similar findings; aged (15- to 18-month old) APOE4 females exhibit deficits in spatial learning and memory in the water maze (Andrews-Zwilling et al., 2010; Bour et al., 2008), although some reports describe impaired spatial recognition, novel object recognition, and contextual fear conditioning in younger APOE4 females (Boehm-Cagan and Michaelson, 2014; Grootendorst et
al., 2005; Segev et al., 2013). In contrast, other reports have described opposing behavioral results in the spatial acquisition phase of the water maze (Siegel et al., 2012; Villasana et al., 2006). Younger (6- to 8-month old) APOE4 mice show enhanced task and spatial learning relative to APOE3 mice, with no apoE isoform-dependent differences in spatial memory. Even in the presence of human APP expression, which would be expected to negatively impact cognition, younger (3-month old) APOE4 mice reportedly exhibit improved spatial learning and memory (Moreau et al., 2013).

These seemingly conflicting behavioral roles for apoE4 have been ascribed to differences in environmental testing conditions among labs, apoE levels in brain regions mediating cognitive behaviors, and differences in anxiety levels in the APOE mice (Siegel et al., 2012). Further investigation into the direct role apoE isoforms play in cognitive behavior are necessary to reconcile these disparities. In addition, more studies utilizing both male and female mice at different ages are warranted.

*Liver X Receptors as a Therapeutic Target in AD*

The liver X receptors (LXR) are ligand-activated nuclear receptors that play a pivotal role in lipid homeostasis in mammals; for a review, see (Hong and Tontonoz, 2014). Upon ligand-binding, LXRs form heterodimers with the retinoid X receptors (RXR), which then bind as LXR/RXR complexes to the liver x receptor response element in the promoter region of certain genes (Sodhi and Singh, 2013). Several of these genes include key regulators of cholesterol efflux, including *ABCA1, ABCG1*, and *APOE* (Abildayeva et al., 2006; Laffitte et al., 2001). In basal states (no bound ligand), the LXR/RXR heterodimer is thought to remain bound on
response elements and complex with co-repressors that inhibit the transcription of target genes (Svensson et al., 2003).

LXR activation has received considerable attention in the AD literature, as a variety of LXR agonists have been shown to increase Aβ clearance and improve cognitive behavior in mice, partly through the upregulation of ABCA1 and apoE (Boehm-Cagan and Michaelson, 2014; Cramer et al., 2012; Jiang et al., 2008; Koldamova et al., 2003; Riddell et al., 2007; Sun et al., 2003). An additional role for LXR agonism in mediating inflammatory responses has also been proposed (Cui et al., 2012; Zelcer et al., 2007), with specific effects observed in microglial cells (Sodhi and Singh, 2013).
C. Long-Term Potentiation

The discovery of post-tetanic potentiation in the hippocampus ushered in a new era of physiological brain research (Bliss and Lomo, 1973). By stimulating monosynaptic fibers at high frequency along the perforant path in anaesthetized rabbits, Bliss and Lomo (1973) were able to record a progressive increase in the amplitude of population spikes from discharging granule cells in the dentate gyrus (DG-LTP). Similarly, a progressive increase in the magnitude of excitatory postsynaptic potentials (EPSPs) could also be recorded in neuronal dendrites following similar stimulation parameters. Long-term potentiation (LTP), a term coined to describe this phenomena (Douglas and Goddard, 1975), can thus be defined as a persistent, use-dependent increase in the efficiency of synaptic transmission (Blitzer, 2005). The induction and maintenance of hippocampal LTP is dependent on the activation of postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors, while a host of neuronal and non-neuronal proteins regulate its modulation.

APOE and its Receptors in LTP

Receptors for apoE have been implicated in LTP processes. ApoER2 plays a functional role in modulating LTP in vitro. LTP recorded in CA1 (CA1-LTP) of wild type slices is abolished upon general inhibition of ligand binding to the LDL receptors by receptor-associated protein (RAP), and recordings from ApoER2−/− slices exhibit diminished CA1-LTP without effecting baseline synaptic transmission or short-term synaptic plasticity (Trommer et al., 2004; Weeber et al., 2002). ApoER2 signaling may influence LTP via interaction with NMDARs at dendritic spines, as an alternatively spliced ApoER2 domain (exon-19) has been shown to couple
ApoER2 to NMDA receptor complexes via PSD-95 (Beffert et al., 2005). Subsequent phosphorylation of disabled 1 (Dab1) and the tyrosine kinase Src may contribute to postsynaptic Ca\(^{2+}\) conductance and downstream signaling processes that affect LTP (Beffert et al., 2005).

ApoE isoforms differentially modulate LTP within in vitro slice cultures; for a review, see Rogers & Weeber (2009). The acute application of recombinant (rec-) -apoE4 enhances CA1-LTP in APOE\(^{-/-}\) slices following theta-burst stimulation (Korwek et al., 2009). In comparison, rec-apoE2 decreases CA1-LTP, while rec-apoE3 has no effect. In slices harvested from young APOE4 mice, both theta burst stimulation and high frequency stimulation of Schaffer collaterals led to enhanced CA1-LTP, mirroring the pattern of effects seen when using recombinant proteins (Kitamura et al., 2004; Korwek et al., 2009). Bath application of the extracellular glycoprotein Reelin (which binds some of the same receptors as apoE) enhances CA1-LTP in slices from APOE2 and APOE3 mice, but not in APOE4 slices (Chen et al., 2010). Activation of NMDA receptor-dependent Ca\(^{2+}\) influx into cultured cortical neurons is antagonized by application of rec-apoE4, but not rec-apoE2 or rec-apoE3 (Chen et al., 2010). In contrast, tetanic stimulation of the medial perforant path results in attenuated DG-LTP in APOE2 and APOE4 slices, with no difference between APOE3 and wildtype slices (Trommer et al., 2004).

These data suggest that apoE isoforms differentially modulate hippocampal LTP in a sub-region dependent manner, and lend credence to the idea that impaired APOE signaling may affect learning and memory processes in the normal brain. A mechanistic understanding of how apoE isoforms differentially influence activity-induced synaptic plasticity is lacking, resulting in an information gap at the neuronal network level.
D. Overview of Topics

All experimental materials and methods utilized during dissertation research are described in Chapter II, with product vendors listed where appropriate. All rodent experiments were conducted in accordance with NIH and institutional guidelines for the care and use of laboratory animals. Chapters III-V describe all experiments performed, with an outline of the hypotheses to be tested and a statistical description of the experimental results. A focused discussion of the results immediately follow the statistical analyses for each chapter. Chapter VI contains a summary and broad discussion of the results, with an emphasis on their impact to our overall understanding of APOE in brain function. I also discuss future directions based on the experimental results, followed by conclusive statements about the information gained through dissertation research in Chapter VII. All published work cited throughout the body of this thesis are hyperlinked and listed in Chapter VIII.
CHAPTER II
MATERIALS & METHODS

A. Animals

APOE Targeted Replacement mice were used for all behavioral experiments and Golgi-Cox staining (hereafter referred to as APOE mice). Briefly, APOE mice express each of the human APOE isoforms under the control of endogenous murine Apoe regulatory sequences and are homozygous for APOE-ε2, APOE-ε3, or APOE-ε4 (Rodriguez et al., 2013; Sullivan et al., 1997). These animals were maintained in a temperature and humidity controlled vivarium at Georgetown University Medical Center, provided food and water ad libitum, and subjected to a standard 12 hr light/dark cycle. Behavioral manipulations were performed during the light phase, while avoiding light/dark transitions. EFAD mice were used for all experiments in Chapter III and have been described previously (Youmans et al., 2012b). Briefly, EFAD mice co-express five FAD mutations (APP K670N/M671L + I716V + V717I and PS1 M1461L + L286V) on the backgrounds of homozygous APOE2, APOE3, or APOE4 targeted replacement genotypes. This cross yielded three lines of mice: E2FAD, E3FAD, and E4FAD. EFAD mice were maintained at the University of Illinois at Chicago in a temperature and humidity controlled vivarium and provided food and water ad libitum. All mice were on a C57BL/6J genetic background.

For multielectrode array experiments described in Chapter V, we cultured primary hippocampal cells from Sprague-Dawley rat pups (embryonic day 18) using a modified protocol from (Pak et al., 2001). Pregnant dams were purchased from Charles River Laboratories.
(Frederick, MD) and maintained at Georgetown University in identical housing conditions to the mice until surgery.

All rodent experiments were conducted in accordance with National Institutes of Health guidelines for the care and use of laboratory animals, and were reviewed and approved by institutional animal care and use committees at Georgetown University Medical Center and the University of Illinois at Chicago.
B. Immunohistochemistry

Tissue Harvesting & Processing

Brain tissue from EFAD mice was harvested as previously described (Youmans et al., 2012b). Briefly, 6-month old EFAD mice were deeply anesthetized with sodium pentobarbital (50mg/kg) and perfused transcardially with ice-cold 100mM phosphate-buffered saline (PBS) pH 7.4. Brains were dissected at the midline, with the left hemi-brains fixed in 4% paraformaldehyde (PFA) for 48 hr, rinsed and stored at 4°C in PBS + 0.05% sodium azide (NaN₃) until tissue sectioning. Right hemi-brains were dissected on ice to yield cortex, hippocampus, and cerebellum samples, then snap frozen in liquid nitrogen, and finally stored at -80°C until use.

Sagittal tissue sections from EFAD hemi-brains (30µm) were sliced in ice-cold PBS using a Leica VT1000S vibratome and stored in cryoprotectant at -20°C until the immunostaining procedures. Tissue sections processed for staining and analysis correspond approximately to plates 111, 115, and 119 in the Paxinos and Franklin mouse brain atlas (Paxinos and Franklin, 2001), following a 1:9 series of tissue staining. For both peroxidase and fluorescence staining procedures, E2FAD, E3FAD, and E4FAD brain sections were processed in parallel.

DAB Immunoperoxidase Staining

The cryoprotectant was washed from free-floating EFAD tissue sections with PBS before quenching endogenous peroxidases with 3% H₂O₂. Sections were then blocked with 3% bovine serum albumin (BSA) and 10% horse serum in PBS for 1 hr at room temperature, followed by
overnight incubation at 4°C with either Iba1 (1:5,000) (WAKO Pure-Chemical Industries, Osaka, Japan) or GFAP (rabbit, 1:500 dilution of 0.4µg/µL stock) (Invitrogen, Carlsbad, CA) antibodies in blocking buffer. Tissue sections were rinsed and incubated with a biotinylated secondary antibody (goat anti-rabbit, 1:1,000 dilution of 1.5mg/mL stock) (VECTA STAIN Elite kit, Vector Labs, Burlingame, CA) for 1 hr at room temperature. Sections were then incubated with an avidin-biotin conjugate (VECTA STAIN Elite ABC Kit, Vector Labs) before being developed in PBS containing 0.04% 3,3’-diaminobenzidine (DAB) hydrochloride and 0.04% nickel ammonium sulfate. After staining was complete, sections were mounted on glass Superfrost Plus slides, allowed to air dry for 24 hr, then dehydrated and cleared with xylene before being coverslipped.

**Immunofluorescence Staining**

Free-floating tissue sections from EFAD mice were washed in tris-buffered saline (TBS) pH 7.4 to remove the cryoprotectant. Sections were then subjected to heat-mediated antigen retrieval for 10 min in 30mM sodium citrate buffer (pH 6.0) containing 0.05% Tween 20. Sections were then permeabilized with 0.25% Triton X-100 in TBS (TBST) and blocked with 5% BSA in TBST for 1 hr at room temperature. Tissue sections were subsequently incubated with an anti-Aβ antibody, MOAB2 (mouse IgG2b, 1:1,000 dilution of 0.5mg/ml stock) (Youmans et al., 2012a; Youmans et al., 2012b), and either an anti-microglia antibody, Iba1 (rabbit IgG, 1:5,000 dilution of 0.5µg/µL stock) (WAKO Pure-Chemical Industries), an anti-astrocyte antibody, GFAP (rabbit IgG, 1:1,000 of stock) (Chemicon-Millipore, Billerica, MA), or an anti-human apolipoprotein E antibody, apoE (goat IgG, 1:5,000 dilution of stock) (Calbiochem-
Millipore, Billerica, MA), diluted in TBST containing 2% BSA overnight at 4°C. In experiments that probed for Aβ and apoE, sequential application of primary antibodies were carried out at 4°C over 48 hr. Next, sections were washed in TBST, then incubated with Alexa fluorophore-conjugated secondary antibodies (Invitrogen, Carlsbad, CA) diluted 1:1,000 in TBST containing 2% BSA for 1 hr at room temperature. After subsequent washing, tissue sections were mounted onto glass Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) using Fluoromount-G (Southern Biotech, Birmingham, AL), a coverslip was added, and the slides were stored in the dark at 4°C until imaging.
C. Golgi-Cox staining

To assess dendritic morphology of MEC neurons described in Chapter IV, brains from naïve 3-month-old APOE TR mice were dissected and processed for Golgi staining using the FD Rapid Golgi Stain kit (FD NeuroTechnologies, Inc., Baltimore, MD). These procedures have been described previously (Dumanis et al., 2009; Hoe et al., 2009). Briefly, we immersed the dissected APOE TR brains in solutions A and B for 2 weeks in the dark at room temperature before transferring to solution C for 48 hr at 4°C. 150μm thick sagittal sections were sliced on a Leica VT1000S vibratome and mounted onto gelatin coated slides (Lab Scientific, Livingston, NJ) and allowed to dry for 48 to 72 hr. Excess solution C was absorbed with filter paper. We noticed that rehydrating the tissue sections in Milli-Q H₂O per the manufacturer’s recommendations led to an increased separation of the sections from the slides. Thus, we reduced this step to one H₂O rinse for 4 min. Sections were then immersed in a mixture containing solutions D and E, rinsed, dehydrated with ethanol, and then cleared with xylene before being coverslipped.
D. Microscopy & Image Analysis

DAB Immunoperoxidase Analysis

Immunoperoxidase-stained EFAD brain tissue was analyzed under bright field microscopy using an AxioPhot upright microscope (Zeiss Microsystems, Inc., Oberkochen, Germany). Digital images were acquired using an AxioCam HRm camera connected to a Dell computer running AxioVision 4.8.2 software (Zeiss Microsystems, Inc.). Region-specific gliosis was observed and qualitatively assessed in the subiculum and deep layers of the cortex by an investigator blinded to APOE genotype. Dystrophic astrocytes were readily identified by their large somas, hypertrophic primary processes and intense GFAP-immunoreactivity relative to astrocytes outside the subiculum and deep layers of the cortex. Reactive microglia were characterized by swollen cell bodies and intense Iba1-immunoreactivity.

Immunofluorescence Analysis

In separate experiments processed for immunofluorescence staining, z-stacked images of EFAD brain sections were acquired and processed into two-dimensional projection images using a Zeiss 510LSM confocal microscope and the LSM Image Browser (Zeiss Microsystems, Inc.), respectively. Image files were coded and then analyzed in Fiji by an investigator blinded to APOE genotype (Schindelin et al., 2012).a

Morphological features of Aβ plaques and gliosis in EFAD brains were analyzed within individual fluorescent channels in Fiji and saved via the ROI Manager. Aβ plaques were defined

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a I wish to thank Ryan Morgan, then a Georgetown University undergraduate, and Bobby Kelly, then a senior at Thomas Jefferson High School, for expert technical assistance in confocal image post-processing and data analysis.
as MOAB2-positive areas of stained tissue consisting of a single massed body at least 10 µm in diameter. Individual plaques within the subiculum and deep layers of the cortex were selected for analysis by an investigator blinded to APOE genotype. Plaques were traced to obtain the plaque area (µm²), fluorescence intensity (AU) and plaque type (Figure 2A and Figure 2B). Sampled plaques were classified into three major categories based on a previous report (Youmans et al., 2012b): (a) diffuse, having no center and weak MOAB2 staining with a wispy morphology; (b) dense core, having an obvious center that stains brightly with MOAB2, and having weakly stained fibrils surrounding the core and (c) compact, having a very brightly stained core with no obvious MOAB2 stained fibrils, and generally smaller than other plaques (approximately 10 to 20µm in diameter). Investigators sampled as many plaques as possible in an image window for analysis, resulting in over 200 plaques traced per APOE genotype. To obtain morphological measures of Aβ-associated microgliosis, 75-µm-diameter rings were centered over plaques to define plaque domains and then superimposed on images of Iba1-stained microglia (Figure 2A and Figure 2C). Plaques with little to no overlapping plaque domains were randomly selected under the same blinded conditions as plaque analysis, as was image analysis of microgliosis. Total Aβ-associated microglial density was calculated within each plaque domain, with a distinct cell soma required for cell counts. Iba1 fluorescence intensities were also calculated within each plaque domain. For each fluorescence channel, three measures of background fluorescence were collected and the average subtracted from the total fluorescence of individual plaques and plaque domains in each image.

Additionally, the extent of Aβ-associated microgliosis in EFAD sections was analyzed by thresholding the images and measuring the total area (µm²) occupied by Iba1-stained microglial
cell bodies and processes within individual plaque domains (Figure 2D). Minimum threshold values for 8-bit single-channel images of Iba1 staining were adjusted interactively (range 35 to 45) under blinded conditions in Fiji using an over/under display mode (blue is background). The aggregate Iba1 immunoreactivity above threshold was then measured within the superimposed plaque domains and saved as a percentage of total plaque domain area.
Figure 2. Confocal microscopy analysis of Aβ-associated microglial activation in EFAD mice. Sagittal brain sections from 6-month old EFAD mice were analyzed for Aβ deposition and associated microglial activation in two regions of interest, the subiculum and deep layers of the cortex. Reactive microglial cells within the vicinity of Aβ plaques consistently exhibited an amoeboid-like shape with dystrophic processes. (A) Two-dimensional projection image (approximately 20µm, z-axis) of an E3FAD subiculum double-stained for Aβ<sub>1-42</sub> (MOAB2) and activated microglia (Iba1). Image overlays depict metrics used to characterize and quantify Aβ deposition and associated microglial activation. White arrows: Examples of plaque domains. (B) Grayscale image of Aβ plaques chosen for analysis. Magenta: Traced plaques. (C) Grayscale image of activate microglia with plaque domain overlays. Yellow rings: Plaque domains (44.2 mm<sup>2</sup>). (D) Thresholding was performed within plaque domains to quantify the percentage of area occupied by microglial cell bodies and processes. Representative minimum threshold value 35 (0 to 255 brightness scale) depicted. Blue: Background. Scale bars: 20µm.
The MEC was identified in 3-4 sections/APOE brain using a mouse brain atlas (Paxinos and Franklin, 2001). A clustered band of Golgi-stained cells were observed in MEC layers II/III, and were delineated by the mostly acellular molecular layer I near the pial surface and the lamina dissecans (layer IV). Only fully impregnated layer II/III pyramidal neurons with clear dendritic arbors were chosen for analysis. High resolution z-stacked images were acquired under bright field microscopy using an Axioplan 2 upright microscope (Zeiss Microsystems, Inc., Oberkochen, Germany) at 63X magnification. The image files were coded and then analyzed by a blinded investigator using the open-source image processing suite Fiji (Schindelin et al., 2012). Total dendritic length was assessed for each neuron by tracing main dendritic processes extending from the soma and their branches. Dendritic segments chosen for spine analysis were randomly selected in a blinded manner from basal and apical arbors. The initial segment (20 µm) of the main apical shaft and apical tufts (near the pial surface) were excluded from analysis.

A representative MEC pyramidal cell was acquired at 40X magnification using an AxioPhot upright microscope (Zeiss Microsystems, Inc.) and is shown with traced overlays to give a sense of dendritic complexity (Figure 3A). Analysis of dendritic length and dendritic spine density was performed on images acquired at 63X only, as high magnification image stacks provided the best clarity with which to delineate dendritic arbors in thick tissue (Figure 3B and Figure 3C).

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*b I wish to thank Dr. Sonya BE Dumanis for expert technical training and assistance during the acquisition of this data. I also thank Dr. Dan TS Pak for generously providing microscope use and technical support for these experiments.*
Figure 3. Dendritic morphology of layers II/III MEC pyramidal neurons in APOE TR mice. Sagittal brain sections from 3-month old APOE mice were processed for Golgi-Cox staining. Dendritic arbors were traced and dendritic spine density was assessed in z-stacked photomicrographs. (A) 40X magnification image in one focal plane of a fully traced MEC pyramidal cell. Traced dendrites (magenta) show complex dendritic arborization. (B, C) 63X magnification images of two individual cells. Basal and apical dendrites were traced in Fiji and the overlays (cyan) were saved via the ROI manager.
E. Behavioral Assays

1. Open Field Test

All mice were transported to the behavioral testing suite in their home cages and allowed to acclimate for 15 min before any further manipulation. Locomotor activity, exploration, and general state anxiety in our young and old APOE mice were assessed using a circular open field maze (880 mm diameter; 55 mm wall height) constructed of white, non-reflective polyethylene plastic (Figure 4). The inner zone was situated in the center of the open field and was bordered by the outer zone, which comprised 54% of the maze area. Testing began by placing the mouse in the center of the inner zone and allowing 300 sec of free exploration. Behavioral activity was recorded via an overhead CCD camera linked to a computer running ANYmaze 4.7 tracking software (Stoelting Co., Wood Dale, IL). Locomotor activity and exploratory drive was assessed via the following parameters: total distance traveled (m), average running speed (cm/sec, and duration of rearing behavior (sec). General state anxiety was estimated using time spent in outer vs. inner zone expressed as a percentage (%) and duration of grooming activity (sec). After each mouse was tested, the maze was thoroughly cleaned with a soap solution followed by water to remove odors. All behavioral testing procedures were conducted by an investigator blinded to APOE genotype.
Figure 4. Illustrated schematic of zones in the OFT. Behavior from our APOE mice was recorded for 300 sec in a circular open field arena, which was divided into an inner zone and an outer zone. We measured the following parameters: total distance traveled, average speed, percentage of time spent in the inner and outer zones, rearing behavior, and duration of grooming.
2. Barnes Maze

Apparatus and Testing Conditions

The circular apparatus used (San Diego Instruments, San Diego, CA) was constructed of white polyethylene plastic (915 mm diameter; 115 cm height) and contained 20 circular holes (50 mm diameter) evenly spaced along the perimeter of the maze. Only the Target Hole (TH) was deep enough for an animal to enter, and led to an escape chamber (108 mm x 55 mm x 55 mm) beneath the maze floor (Figure 5). The remaining nineteen holes in the maze were considered False Holes and were not deep enough for the animals to fully enter or escape the maze. Testing procedures and distal visual cues were modified to enhance visuospatial learning and memory (O’Leary and Brown, 2012). We used a weak aversive auditory stimulus (WAAS) (78 dB blow-dryer simulation, Columbia River Entertainment, 2001) and bright overhead lighting (10.8 x 10^2 lux) to drive escape motivation. We also placed visual cues on the floor of the testing suite, as several animals peered over the edge of the maze in pilot experiments. 3-month old (16 female/19 male) and 18-month old (12 female/15 male) TR mice completed three phases of Barnes Maze testing: habituation, acquisition, and probe.
Figure 5. Barnes maze apparatus, dimensions, and testing conditions. All training and probe trials in the Barnes maze were recorded via an overhead CCD camera. A blinded investigator observed the recorded behavior from an adjacent room. (A) The circular Barnes maze was situated in the center of the behavioral suite. Visual cues in the extra-maze environment were of high contrast in order to maximize usefulness as landmarks for spatial navigation. (B) False holes were any shallow holes that would not allow adult APOE mice to enter into and escape the maze. Depth was ~20mm. There were 19 total. (C) Contrast in dimensions between the False, left, and Target Hole, right. The Target Hole measured 108mm x 55mm x 55mm.
**Habituation Phase**

At least twenty-four hours after open field testing, animals were habituated to the Barnes Maze testing conditions and allowed to freely explore the apparatus for 120 sec before being gently guided to the TH. Mice were given an additional 120 sec inside the escape chamber before being removed and returned to their home cages. The escape chamber was cleaned and inspected for debris after each animal to ensure no lingering odors remained that would influence the next mouse.

**Spatial Acquisition (Learning) Phase**

Twenty-four hours after habituation, APOE mice learned over four days (4 trials/Training Day; inter-trial interval = 15 min; 16 trials total) to use distal visual cues to locate the TH and escape the maze. At the beginning of a trial, animals were placed under a start box in the center of the maze for 10 sec before the start box was removed. A trial was terminated when the mouse had entered the TH or when 180 sec had elapsed. The position of the TH was randomly assigned and differed between habituation and spatial acquisition phases as well as between age-groups (Chapter IV). Latency to escape the maze (sec) was calculated to assess spatial learning, while total distance traveled (m) and average speed (cm/sec) were calculated to detect differences in locomotor activity. The number of hole investigations (total nose pokes) along the perimeter were counted as an indicator of exploration during the training trials. Importantly, the entire maze was thoroughly cleaned with a soapy solution followed by water after each training trial.

**Probe (Memory) Phase**
Twenty-four and seventy-two hours after the last training trial, the TH was closed and spatial memory was assessed in 90 sec probe trials by measuring the latency to investigate the closed TH (Primary Latency). Speed (Primary Speed) and number of investigations of false holes before reaching the TH (Primary Errors) were assessed in addition to total nose pokes. After each probe trial, the Barnes maze was thoroughly cleaned with a soap solution followed by water to remove lingering odors that may influence the next animal.
3. Hidden Platform Water Maze

Apparatus and Testing Conditions

The hidden platform water maze (HPWM) was conducted in the same behavioral suite as the Barnes maze and utilized the same distal visual cues on the walls. The apparatus consisted of a large tank (1.22 m diameter) filled with opaque water maintained at 25°C, with a hidden platform (101 mm diameter) submerged just below the surface. The area of the maze was broken up into quadrants, and the location of the hidden platform was randomly assigned within one of the quadrants. This location remained constant throughout training trials but was switched during visible platform trials at the end of testing.

Spatial Acquisition (Learning) Phase

Naïve 3-month old APOE mice were trained over the course of four days (4 trials/Training Day; ITI = 20 min; 16 trials total) to locate the position of the hidden platform. Mice were introduced into the pool and tracked during a 90 sec training trial using the ANYmaze system. A training trial was terminated once the animal located the platform and remained on it for 2 sec. The animal was subsequently removed from the platform after 60 sec, gently dried with a cloth, and returned to a holding cage. If an animal did not successfully find the hidden platform, it was gently guided to the platform and allowed to remain there for 60 sec before being removed and dried. Latency to find the hidden platform (sec), distance traveled (m), and
average speed (cm/sec) were calculated to assess spatial learning behavior and locomotor activity over TDs.

Probe (Memory) Phase

Seventy-two hours after the last training trial, the hidden platform was removed from the maze and long-term spatial memory was assessed in a single 90 sec probe trial. The average time spent in the Target Quadrant was recorded as well as the number of platform site crossings.

Visible Platform Phase

One hour after the Probe Trial, APOE mice were tested for visual acuity in the water maze using a visible platform placed in a new quadrant each trial. Latency to reach the platform, distance traveled, and swim speed were recorded in each of 3 trials (90 sec; ITI = 20 min).

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*I wish to thank Bryan Epley, then a Georgetown University Howard Hughes Scholar and United States Army veteran, for his assistance in hidden platform water maze training during the Fourth of July weekend. He certainly earned and deserved the time off, but chose to assist in the experiments anyway.*
F. Primary Hippocampal Cultures

Tissue Harvesting/Dissociation

Primary hippocampal cells were harvested from embryonic day 18 (E18) Sprague Dawley rat pups and cultured as previously described (Niedringhaus et al., 2013; Pak et al., 2001). Briefly, hippocampi were dissected from E18 rat pups in ice-cold dissecting solution (Table 1), with the meninges being carefully removed from the extracted tissue. Pooled hippocampi were then washed, trypsinized (0.25%) for 15 min at 37°C, exposed to 0.001% DNAse at room temperature for 5 min, and then washed with pre-warmed maintenance media (Table 1). The tissue was then sequentially triturated with two sterilized Pasteur pipets. First, we used a fire polished pipet (smoothed tip edges) at normal tip diameter, followed by a fire polished pipet in which the tip diameter had been reduced. Dissociated hippocampal cells were then immediately plated onto prepared glass coverslips or multielectrode arrays.
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Table 1. Medium Composition for Cell Culture Experiments. Hippocampal tissue from E18 Sprague Dawley rat pups was dissected in ice-cold Dissection media composed of HBSS, Sodium Pyruvate, D-Glucose, HEPES, and Pen/Strep at a working concentration. Dissociated hippocampal cells were then plated onto our substrates in pre-warmed Maintenance media composed of Neurobasal A, GlutaMAX, B-27, and Pen/Strep at working concentrations. As needed, fresh Maintenance media was replaced or supplemented into the cultures.
Culture Maintenance and LXR Agonist TO901317 Treatment

Dissociated hippocampal cultures were maintained in Neurobasal medium supplemented with B27 in a humidified 5% CO₂ and 95% O₂ incubator at 37°C. Approximately five hours after plating, cultures were inspected for proper adhesion of the cells to either the glass coverslips or multielectrode array floor. Cells in culture were allowed forty-eight to seventy-two hours in the incubator before being removed and inspected for healthy cell growth. Cultures showing signs of contamination were immediately discarded and the incubator cleaned. For electrophysiology experiments, multielectrode arrays were capped with a semi-permeable Teflon lid (Multi-Channel System) to maintain sterility, osmolality, and media pH within the recording chamber (Potter and DeMarse, 2001). In all of our primary culture experiments, pre-warmed maintenance media was either supplemented or replaced as needed to ensure the health of the high-density neuronal networks.

Mature hippocampal cells (DIV14-15) were treated with the LXR agonist TO-901317 (Cayman Chemical, Ann Arbor, MI) for 120hr at 37°C. Briefly, a 20μM concentration of TO-901317 was prepared from a 2.5mM stock solution by diluting the stock in pre-warmed maintenance media. This solution was then further diluted into a working concentration (1μM or 5μM) of drug by exchanging a small volume of conditioned media (CM) in the cultures with pre-warmed media containing drug. Vehicle-treated cultures were prepared in a similar manner by exchanging CM with dimethyl sulfoxide (DMSO, working dilution 1:1,000) (Sigma-Aldrich, St. Louis, MO). Non-treated hippocampal cultures underwent a media exchange only before being returned to the incubator.
G. Western blot

Sample Preparation

CM samples from individual MEAs were pooled on DIV12 and DIV15. CM from DMSO, 1μM, or 5μM treated MEAs were pooled on DIV20 and DIV25. Samples were immediately spun at 2,500 rpm for 3 min in a temperature controlled centrifuge and the supernatants collected. Samples were prepared with 4X NuPAGE LDS loading buffer (Life Technologies, Carlsbad, CA), reduced with beta-mercaptoethanol and boiled for 5 min before being loaded into polyacrylamide gels.

Gel Electrophoresis and Immunoblotting

CM samples were loaded in equal volume and electrophoresed on 10% polyacrylamide gels at 110 mV for approximately 1.5 hr. The separated proteins were then transferred to nitrocellulose membranes (BioRad, Hercules, CA) and stained with Ponceau to ensure proper transfer. Membranes were then washed with heated TBS-T for 5 min before being blocked with 10% milk, and then incubated with anti-apoE primary (rabbit polyclonal, 20487: AbCam, Cambridge, England) and left overnight at 4°C. Following secondary antibody incubation, membranes were developed with SuperSignal West PICO chemiluminescent substrate (Thermo Scientific, Rockford, IL) for approximately 3 min and developed using HyBlot ES autoradiographic film (Denville Scientific, South Plainfield, NJ). Developed films were then scanned and saved as TIFF files on a personal Dell Computer.
H. Multielectrode Arrays

Substrate Preparation

Pre-cleaned, sterilized multielectrode arrays (MEA) (Multi-Channel Systems, Reutlingen, Germany) were coated overnight with Poly-D-lysine hydrobromide (1mL, 0.1mg/mL) (Sigma-Aldrich, St. Louis, MO), washed the next morning with PBS, and then coated with laminin (Roche Diagnostics, Indianapolis, IN) at a working concentration of 2-5 µg/cm². The next morning, MEAs were again washed with PBS and then incubated at 37°C with fresh maintenance media for at least an hour before plating cells. Parallel experiments were run for immunocytochemistry (ICC) using dissociated hippocampal cells from the same harvested tissue. Hippocampal cells were plated onto 15mm borosilicate hydrolytic class I German glass coverslips, which were cleaned and coated identically to our MEAs.

MEA Recordings

All electrophysiological recordings using the MEA system took place in the Neural Dynamics Laboratory. The MEA is composed of 59 titanium nitride electrodes, arranged on an 8x8 grid, with one internal reference electrode and four auxiliary analog channels. Each electrode is 30µm in diameter and the inter-electrode spacing is 200µm. Spontaneous or drug-induced electrical activity from the networks was amplified and sampled at a rate of 10 kHz in order to allow for the detection of spiking activity. During recording, MEAs were kept on a heated stage to maintain 37°C temperature and capped with a semi-permeable Teflon lid (Multi-Channel Systems) (Potter and DeMarse, 2001). Electrophysiological data was digitized and stored on a personal Dell computer for offline analysis.
Data Analysis

Raw .MCD files were replayed in MC_RACK (Multi-Channel Systems) and high-pass filtered at 200Hz before being imported into Plexon Offline Sorter v3 (Dallas, TX). Extracellularly recorded spikes were detected using a threshold algorithm, which was calculated as a multiple of the standard deviation (range of -4.5 to -5σ) of biological noise in our cultures (Niedringhaus et al., 2012; Niedringhaus et al., 2013). The extracted timestamps for spiking activity in each channel were then exported into MATLAB (MathWorks, Natick, MA) for further analysis. To assess overall spiking activity in our hippocampal networks, we calculated the total number of spikes detected in the 15 min recording sessions (DIV150 and DIV20). We also calculated the number of bursts in our networks, which we defined as periods of high frequency spiking activity that consisted of no less than 4 spikes with a maximum inter-spike interval. A maximum inter-spike interval for calculating bursts was selected after examining log-scaled histograms of frequency counts of inter-spike intervals (in milliseconds) (Origin 8.0, Northhampton, MA). In addition, we calculated the average burst durations (ms), the average number of spikes within bursts, and the inter-spike intervals within bursts.
I. Statistical Analysis

All statistical analyses were performed using GraphPad Prism 5 unless noted otherwise. Differences between means were assessed by \textit{t}-test, one-way analysis of variance (ANOVA), two-way ANOVA, or two-way ANOVA with repeated measures, followed by Tukey’s honest significant difference, Bonferroni multiple comparisons, or Dunnett’s multiple comparisons \textit{post hoc} tests where appropriate. Descriptive statistics are expressed as mean ± standard error of the mean, with significance set at $P < 0.05$. 
CHAPTER III

EFFECTS OF APOE GENOTYPE ON Aβ PLAQUES & MICROGLIAL REACTIVITY.

Figure 6. Image of Aβ-associated microglial reactivity selected for Journal of Neuroinflammation article cover. Double immunofluorescence staining for Aβ1-42 (cyan) and activated microglia (red) in the subiculum of a 6-month-old E3FAD mouse brain section. Microglia that surround the Aβ deposits exhibit swollen cell somas, intense Iba1 immunoreactivity, and dystrophic processes compared to areas with no Aβ plaques. Scale bar: 30 μm.

This chapter is modified from Rodriguez GA, Tai LM, LaDu MJ, Rebeck GW. (2014). Human APOE4 increases microglia reactivity at Aβ plaques in a mouse model of Aβ deposition. Journal of Neuroinflammation. 11:111. (PMID: 24948358)
A. Overview

The experiments described in Chapter III were designed to test the general hypothesis that APOE genotype affects Aβ-associated neuroinflammation in the mouse brain. Specifically, I was interested in assessing whether APOE genotype would influence astrocyte and/or microglia reactivity at individual Aβ plaques in the EFAD brain. First, I describe Aβ plaque profiles in the subiculum and deep layers of the cortex. I then describe Aβ-associated microglial reactivity in these regions by looking at markers of inflammatory microglia within plaque micro-environments. These analyses shift the focus away from global measures of gliosis, which could be influenced by effects of APOE genotype on overall plaque burden, to Aβ plaque-associated neuroinflammatory markers. These data are the first to address the synergistic effect of APOE genotype and Aβ plaques on microglia reactivity in these mice. However, more research on Aβ deposition and plaque-associated neuroinflammation in the EFAD model is needed, with an emphasis on microglial-secreted pro-inflammatory and anti-inflammatory signaling molecules that affect nearby cells.
B. Experimental Results

1. APOE Genotype Affects Aβ Plaque Profiles in the EFAD Brain

To investigate the effects of APOE genotype on Aβ deposition and Aβ plaque-associated neuroinflammation, we performed experiments using double immunofluorescence staining for Aβ and microglia on tissue sections from 6-month EFAD mice (E2FAD, n=4; E3FAD, n=5; E4FAD, n=5). EFAD mice exhibit region-specific accumulation of extracellular Aβ plaques in the subiculum and deep layers of the cortex. Thus, we initially focused on assessing Aβ deposition in these regions by measuring Aβ1-42 (MOAB2) immunoreactivity and comparing Aβ plaque profiles among APOE genotypes (Youmans et al., 2012b). We quantified the following parameters from selected, individual Aβ-positive deposits in the subiculum and deep layers of the cortex: plaque area, fluorescence intensity, and plaque type.

In the subiculum, we found a significant effect of APOE genotype on plaque size ($F_{(2,11)} = 4.30$, $P<0.05$), with post hoc comparisons revealing significantly larger plaques sampled in E4FAD sections compared to E2FAD sections ($P<0.05$) (Figure 7B, left). MOAB2 fluorescence intensity was not affected by APOE genotype ($F_{(2,11)} = 3.25$, $P<0.078$) in this brain region (Figure 7B, center). Finally, we defined Aβ deposits as diffuse, dense core, or compact plaques and expressed the total number of diffuse, dense core and compact plaques as a percentage of total plaques, and compared them within and among APOE genotypes (Figure 7A and Figure 7B, right). Two-way ANOVA revealed a significant main effect of plaque type ($F_{(2,33)} = 26.65$, $P<0.001$) on the distribution of plaques in all EFAD groups, with a greater number of dense core (45.83 ± 0.3%) plaques present in the subiculum than diffuse plaques (32.93 ± 0.5%) or compact
plaques (21.10 ± 0.5%). There were a higher percentage of diffuse plaques in E2FAD sections compared to E3FAD sections (P<0.05) (E2FAD vs E3FAD; 45.00 ± 3.79% vs. 28.30 ± 2.83%) and E4FAD sections (25.50 ± 3.48%) (P<0.01) (Figure 7B, right). There were also significantly fewer dense core plaques in E2FAD sections (33.90 ± 2.59%) than E3FAD sections (57.60 ± 2.83%) (P<0.01), and a higher percentage of compact plaques in E4FAD sections (28.50 ± 4.80%) than E3FAD sections (13.90 ± 1.08%) (P<0.05). Thus, in the subiculum, APOE2 was associated with more diffuse plaques, and APOE4 with more compact plaques, compared to APOE3.

Within the deep layers of the cortex, we did not detect EFAD group differences in plaque size ($F_{(2,11)} = 3.45, P=0.068$) (Figure 7C, left). However, APOE genotype did affect MOAB2 fluorescence intensity ($F_{(2,11)} = 4.88, P<0.05$), with E4FAD plaques exhibiting higher MOAB2 positive staining compared to E3FAD plaques (P<0.05) (Figure 7C, center). A two-way ANOVA to assess the distribution of plaque types in the cortex revealed a significant main effect of plaque type ($F_{(2, 33)} = 21.43, P<0.001$) for each EFAD group, with higher percentages of diffuse plaques (42.74 ± 4.99%) and dense core plaques (35.79 ± 3.74%) present in EFAD sections than compact plaques (21.70 ± 1.94%) (Figure 7C, right). Interestingly, a higher percentage of diffuse plaques were again observed in E2FAD sections (50.80 ± 3.61%) compared to E4FAD sections (33.61 ± 5.05%).

These data examine extracellular Aβ plaques in two sites of robust Aβ deposition in the brains of 6-month-old EFAD mice. APOE genotype had an effect on plaque characteristics in both the subiculum and deep layers of the cortex, which is consistent with previous findings for these mice (Youmans et al., 2012b).
Figure 7. APOE genotype affects Aβ plaque morphology in EFAD subiculum and deep layers of the cortex. Morphologically distinct Aβ plaques were visualized in the subiculum and deep layers of the cortex of 6-month old EFAD brain sections using immunofluorescence staining with Aβ antibody MOAB2. (A) Heterogeneous plaque types were evident in EFAD sections and grouped into three categories: diffuse, dense core and compact. Scale bar: 12 µm. (B) Subiculum. Left: E4FAD sections exhibited larger plaques on average than E2FAD sections. Center: Fluorescence intensity of plaques did not differ by APOE genotype. Right: E2FAD sections exhibited a greater percentage of diffuse plaques than E3FAD or E4FAD sections, and a lower percentage of dense core plaques than E3FAD sections. Interestingly, E4FAD sections exhibited an increased percentage of compact plaques in the subiculum. (C) Deep layers of the cortex. Left: Plaque size did not differ by APOE genotype. Center: Fluorescence intensity of deep cortical plaques was highest in E4FAD sections. Right: E2FAD sections had a greater percentage of diffuse plaques in this region than E4FAD sections. No differences were detected between APOE genotypes for dense core or compact plaques. One-way ANOVA, * P<0.05. Two-way ANOVA, * P<0.05, ** P<0.01.
2. Region-Specific Glial Activation

Aβ accumulation and deposition in the brain can lead to reactive gliosis, which refers to the physiological activation of glial cells in response to focal tissue damage, and is characterized by specific structural and functional changes to glial cells that mediate the neuroinflammatory response (Burda and Sofroniew, 2014). Astrocytes and microglia are two important glial cell types involved in Aβ-associated glial activation (Birch et al., 2014). Therefore, we assessed glial activation in 6-month-old EFAD mice by analyzing the distribution and numbers of dystrophic astrocytes and microglial cells in the brain.

DAB-immunoperoxidase staining for GFAP (astrocytes) was performed on sagittal brain sections from E2FAD, E3FAD and E4FAD mice. Intense GFAP staining was evident in the subiculum of all EFAD mice (Figure 8A), with numerous reactive astrocytes having dystrophic processes in the region (Figure 8A, inset). Sparse, but intense, GFAP staining was also evident in deep layers of the cortex, where clusters of reactive astrocytes were detected (Figure 8A, magenta arrows). Double immunofluorescence staining for GFAP and MOAB2 confirmed that plaques were at the center of reactive astrocytic clusters in deep cortical layers (data not shown). DAB staining for Iba1 revealed a similar pattern of immunoreactivity for microglia in the EFAD subiculum, although reactive microglia appeared more frequent in the E4FAD and E2FAD cortices than in E3FAD cortices (Figure 8B, green arrows). Double immunofluorescence staining for reactive astrocytes and microglia was performed on adjacent EFAD sections to visualize reactive gliosis in these regions. Dystrophic astrocytes and microglial reactivity were clearly evident in the subiculum, with both glial cell types exhibiting strong intensity and activated morphologies (Figure 8C).
Figure 8. Glial activation is increased in the EFAD subiculum and deep layers of the cortex. Astrocytes and microglia were visualized in sagittal brain sections of 6-month old EFAD mice using IHC. Initial DAB staining revealed prominent gliosis in two regions of the brain: the subiculum and deep layers of the cortex. (A) Extensive GFAP-immunoperoxidase staining was evident in the subiculum of all EFAD mice. In addition, dystrophic astrocytes could be seen throughout the deep layers, but not in superficial layers, of the cortex. Insets: 20X magnification of the subiculum (dashed red box). Magenta arrows: Clusters of dystrophic astrocytes in the cortex. Scale bar: 500µm. (B) Activated microglial cells were clearly visible in the subiculum of all EFAD mice and were present in deep cortical layers as well. E4FAD sections exhibited more activated microglia in the deep cortex than E2FAD and E3FAD sections. Green arrows: Activated microglia. Scale bar: 500µm. (C) Double immunofluorescence staining for GFAP (magenta) and Iba1 (green) confirmed region-specific glial activation in EFAD brains. Representative images of EFAD subiculum are shown. Scale bar: 30µm.
IL-1β is a member of the interleukin-1 family of cytokines and is an important microglia-derived mediator of neuroinflammation. Six-month old 5xFAD mice show significant activation of IL-1β in the brain, suggestive of an Aβ-dependent neuroinflammatory response (Hillmann et al., 2012). APOE genotype exerts influence over inflammatory responses in the brain independent of AD pathology (Wolf et al., 2013). APOE4 is associated with upregulated gene expression of pro-inflammatory cytokines following traumatic brain injury and lipopolysaccharide (LPS) treatment compared to APOE3 (Laskowitz et al., 2010; Zhu et al., 2012). To test whether APOE genotype affects functional aspects of glial activation in EFAD mice, we measured levels of the pro-inflammatory cytokine IL-1β in TBS soluble fractions of 6-month old EFAD cortices (E2FAD, n=4; E3FAD, n=4; E4FAD, n=4) (Figure 9). One-way ANOVA revealed a significant effect of APOE genotype ($F_{(2,9)} = 12.66, P<0.01$) on IL-1β levels. A significant elevation in IL-1β was detected in the E4FAD cortex (41.90 ± 1.90 pg/mg), representing an increase of nearly two-fold compared to E3FAD samples (23.85 ± 1.46 pg/mg) and E2FAD samples (26.05 ± 4.14 pg/mg).

Thus, morphological characteristics of reactive gliosis were revealed in the adult EFAD subiculum and deep layers of the cortex. Initial DAB-immunoperoxidase staining showed strong glial immunoreactivity in the subiculum of all mouse sections. Increased clusters of astrocytes and an increased number of reactive microglial cells were found in deep layers of E4FAD cortex compared to the E3FAD cortex. Z-series projections of double-stained images revealed highly dystrophic astrocytic and microglial processes. In addition, we found elevated levels of pro-inflammatory IL-1β in E4FAD cortex.
Figure 9. E4FAD mice exhibit elevated IL-1β levels in cortex. Levels of the pro-inflammatory cytokine IL-1β were measured using ELISA in 6-month-old EFAD cortex extracts. E4FAD samples exhibited significantly higher levels of IL-1β than E3FAD samples. One-way ANOVA, **P<0.01.

*ELISA data was generated and analyzed at the University of Illinois at Chicago by Dr. Leon M Tai, then a post-doctoral fellow in the laboratory of Dr. Mary Jo LaDu.
3. Aβ-Associated Microglial Reactivity

The Subiculum

Our region-specific glial staining and ELISA data revealed increased microglial reactivity in the E4FAD mouse brain. This may have been due to an effect of APOE on plaque load, or total number of plaques, in the brain (Youmans et al., 2012b). To determine whether APOE genotype had an effect on Aβ-associated microglial reactivity independent of plaque load, we analyzed microglial activation within the vicinity of individual Aβ plaques. Using the MOAB2 and Iba1 double-stained tissue sections (Figure 2 and Figure 7), we established domains around select plaques possessing little to no overlap with other domains. We then isolated the Iba1-positive fluorescence images from the original double-stained sets, superimposed plaque domains on these single-channel images, and quantified the following parameters within plaque domains: average number of microglial cells, Iba1 fluorescence intensity and percent area occupied by microglia (Figure 2C, D).

Representative images of Aβ-associated microglial reactivity are shown for E2FAD, E3FAD, and E4FAD subiculum (Figure 10A). Within this region of the E4FAD brain sections, we did not detect differences in the average number of microglial cells surrounding plaques ($F_{(2, 11)} = 2.64, P>0.05$), Iba1-positive fluorescence intensity ($F_{(2, 11)} = 0.54, P>0.05$) or percent area of plaque domain occupied by microglia ($F_{(2, 11)} = 0.64, P>0.05$) (Figure 10B).
Figure 10. Aβ deposition in the subiculum is associated with high levels of microglial reactivity. Double immunofluorescence staining of 6-month old EFAD brain sections revealed significant Aβ deposition and elevated microglial activation in the subiculum. (A) Representative images of fluorescence staining in EFAD subiculum. Aβ-associated microglial activation was assessed for each APOE genotype (n=4 or 5 mice/APOE genotype). Scale bar: 30µm. (B) Microglial density, fluorescence intensity and percentage area of the plaque domain did not differ between APOE genotypes. Within the subiculum, the reactive microglia in each group occupies a large percentage of the plaques domains relative to the deep cortex. One-way ANOVA, * P < 0.05, ** P < 0.001.
Deep Layers of the Cortex

In deep layers of the cortex, activated microglial profiles were elevated in E4FAD brain sections compared to E3FAD sections (Figure 11A). We found a significant effect of APOE genotype on the average number of microglial cells surrounding plaques ($F_{(2,11)} = 7.90, P<0.01$), with E4FAD sections exhibiting a 2.3-fold increase compared to E3FAD sections ($P<0.01$) (Figure 11B, left). Interestingly, Aβ-associated microglial cell counts were also elevated within E2FAD plaque domains compared to E3FAD plaque domains ($P<0.05$). In addition, differences in Aβ-associated microglia intensity ($F_{(2,11)} = 4.36, P<0.05$) were detected between EFAD groups, with increased staining intensity present in E4FAD plaque domains relative to E3FAD plaque domains ($P<0.05$) (Figure 11B, center). When measuring the percentage area of plaque domains occupied by microglia, we found a significant effect of APOE genotype ($F_{(2,11)} = 7.35, P<0.01$) (Figure 11B, right). Post hoc tests revealed a higher percentage of dystrophic microglia occupying E4FAD plaque domains than E3FAD plaque domains ($P<0.01$). The E2FAD and E3FAD sections analyzed had similar percentages of total microglial staining within plaque domains (E2FAD, 20.0 ± 1.19% vs E3FAD, 16.9 ± 0.83%), despite E2FAD plaque domains containing more microglial cells than E3FAD plaque domains (Figure 11B, left).

With this detailed analysis of EFAD brains, we found that Aβ-associated microglia reactivity was not affected by APOE genotype in the subiculum. However, we found that APOE genotype affected morphological characteristics of microglial reactivity, with several measures of increased microglial activity detected within E4FAD cortical plaque domains, independent of overall plaque load.
Figure 11. APOE4 is associated with increased microglial reactivity at Aβ plaques in deep layers of the cortex. Double immunofluorescence staining of 6-month old EFAD brain sections revealed reactive microglial cells around Aβ plaques in deep layers of the cortex. (A) Representative double-stained images of Aβ plaques and microglial activation in deep layers of EFAD cortices (n=4 to 5 mice/APOE genotype). Scale bar: 30µm. (B) Left: E4FAD and E2FAD sections exhibited increased reactive microglial density within Aβ plaque domains. Center: Fluorescence intensity of the microglial cells was elevated in E4FAD sections relative to E3FAD sections. Right: E4FAD microglia occupied a larger percentage of plaque domains than E3FAD microglia. One-way ANOVA, * P<0.05, **P<0.01.
C. Discussion

APOE-dependent differences in the innate inflammatory response to Aβ may partially explain the differential risk for AD caused by APOE genotype (Kim et al., 2009). In these experiments, we investigated whether APOE alleles affect glial markers of Aβ-associated neuroinflammation in a mouse model of Aβ deposition. First, we found that E4FAD mice exhibited larger, more intensely stained Aβ plaques in the brain compared to E2FAD and E3FAD mice. E4FAD mice also had a greater number of compact plaques in the subiculum than E3FAD mice. Initial immunostaining experiments revealed activated astrocytes and microglial cells in the subiculum and deep layers of the cortex, and an ELISA revealed significantly elevated levels of IL-1β in the E4FAD cortex. These findings prompted our investigation into microglial reactivity surrounding plaques in the subiculum and deep cortex. We did not detect APOE-dependent differences in microglial reactivity within the subiculum, perhaps related to the very intense deposition of Aβ in the region for all groups. In the deep layers of the cortex, however, APOE genotype had a significant effect on Aβ-associated microglial activation. E4FAD mice exhibited higher numbers of reactive microglial cells surrounding Aβ plaques and increased signs of activation compared to E3FAD mice. These data support the idea that Aβ-associated microglial activation, an important component of the neuroinflammatory response in AD, is augmented by the APOE4 genotype.

5xFAD mice exhibit rapid amyloid deposition and pathology in the brain. Extracellular amyloid can be detected as early as 2- to 4-months of age in the subiculum and deep layers of the cortex (Oakley et al., 2006; Youmans et al., 2012b). By introducing human APOE alleles into the 5xFAD model, the effects of APOE on amyloid-associated pathological changes in the adult
brain can be investigated. The EFAD mouse model is particularly good for examining the effects of APOE genotype on Aβ-associated inflammation. In our experiments, we were first interested in characterizing extracellular Aβ plaques in the brains of adult EFAD mice. We found mostly large Aβ plaques in the E4FAD subiculum, with no group differences in MOAB2 staining intensity (Figure 7B). Our morphological results support findings showing a higher percentage of compact plaques in the subiculum of 6-month old E4FAD mice compared to E3FAD mice (Figure 7B) (Youmans et al., 2012b). We also found a higher percentage of diffuse plaques in the subiculum of E2FAD mice compared to E3FAD mice, though no differences were found between these two groups previously (Youmans et al., 2012b). This is likely due to the antibody used to stain plaque deposits. Here we used MOAB2, a pan-specific Aβ antibody that recognizes several conformational species of Aβ$_{1-42}$ (Youmans et al., 2012a) while the previous study used thioflavin-S to stain beta-sheet-rich amyloid fibrils in plaques (Youmans et al., 2012b). MOAB2-stained Aβ plaques were clear and could easily be distinguished from one another using our methods (Figure 7A). Importantly, the percentage of diffuse plaques in the E2FAD subiculum was greater than in the E4FAD subiculum, which supports previous plaque morphology data for these mice (Youmans et al., 2012b). In deep layers of the cortex, APOE genotype did not influence plaque size, though we did detect increased MOAB2-staining intensity in E4FAD plaques compared to E3FAD plaques (Figure 7C). The significance of greater plaque fluorescence intensity on glial activation is unclear, but may be related to changes in microglial reactivity surrounding plaques that we observed in our studies. More research on Aβ deposition and plaque-associated neuroinflammation in the EFAD model is needed, with an emphasis on
microglial-secreted pro-inflammatory and anti-inflammatory signaling molecules that affect nearby cells.

Microglial cells are the resident macrophages in the CNS and are responsible for several functions of the innate immune response (Prokop et al., 2013; Tambuyzer et al., 2009). Notably, the microglia survey the brain parenchyma for foreign substances and cellular debris, shifting their activation states to phagocytose the material and clear the microenvironment. In our experiments, both DAB-immunoperoxidase and double immunofluorescence staining revealed aggressive astrocytosis and microgliosis in the subiculum of E2FAD, E3FAD and E4FAD mice (Figure 8A, C), while reactive microglia were most visible in the cortex of E4FAD and E2FAD mice (Figure 8B). Age-matched cortical samples were analyzed for total IL-1β levels by ELISA. We detected increased levels of pro-inflammatory IL-1β in the E4FAD cortex compared to the E3FAD cortex (Figure 8D). These data led us to investigate morphological markers of microglial reactivity proximal to Aβ plaques in both brain regions. Reactive microglia in AD brains have been found to be localized to Aβ plaques (Itagaki et al., 1989; McGeer et al., 1987), and in vitro studies have shown that Aβ directly activates the microglia to produce IL-1β, reactive oxygen species and tumor necrosis factor α (TNFα) (El Khoury and Luster, 2008; El Khoury et al., 2003; Meda et al., 1995). Interestingly, APOE is associated with Aβ-independent immunomodulatory functions in vitro. ApoE suppresses the LPS-stimulated release of TNFα in primary glial cultures, and attenuates microglial activation by secreting derivatives of amyloid precursor protein in an isoform-dependent manner (Barger and Harmon, 1997; Laskowitz et al., 1997). The anti-inflammatory effects of APOE may be mediated through LRP1 on microglia and subsequent suppression of the c-Jun N-terminal kinase pathway (Pocivavsek et al., 2009a;
Pocivavsek et al., 2009b). In animal models, apoE deficiency is associated with poor recovery from CNS injuries involving neuroinflammation, demonstrating its important immunomodulatory role in the brain (Fagan et al., 1998; Lomnitski et al., 2000). Our experiments using the EFAD model of Aβ deposition have provided additional insights on the role APOE plays in the neuroinflammatory response to AD pathological brain changes.

Analyzing morphological markers of microglial activation within defined Aβ domains allowed us to control for the effects of APOE on amyloid accumulation. Since APOE4 is associated with higher levels of amyloid deposition in both humans and mouse models, higher levels of global inflammation would be expected for mice with APOE4. In our analysis, we measured microglial reactivity within the microenvironment of randomly selected Aβ plaques (Figure 2). To fit our selection criteria, plaques needed to be clearly visible, larger than 10μm in diameter and easily discerned from other plaque types. Every effort was made to select individual plaques for analysis that were at least 35μm from another plaque, which we determined was the maximum distance for which we could accurately judge plaque-specific microglia reactivity. This analysis proved challenging in the subiculum, where each EFAD group exhibited aggressive Aβ deposition, and thus plaques were more likely to be near one another. Our results describing Aβ-associated microglia reactivity in the subiculum may reflect this, as we did not detect APOE genotype differences in Aβ-associated microglia reactivity (Figure 10). We hypothesize that measuring Aβ-associated microglial reactivity in the subiculum of younger EFAD mice would remove the effects of proximal Aβ plaques, as Aβ deposition would be reduced in the region and more accurate assessments of glial activation could be made. Nonetheless, we were able to measure markers of microglia reactivity readily within the
microenvironment of plaques found in the deep layers of the cortex (Figure 11). We detected an increased density of reactive intensely stained microglia surrounding individual plaques in E4FAD sections compared to E3FAD sections. Interestingly, E2FAD mice also exhibited increased numbers of microglial cells surrounding plaques, though these cells did not appear as activated as those from E4FAD mice. This conclusion is reflected in the low Iba1 fluorescence intensity and a smaller percentage area of the plaque domain occupied by cell soma and processes. Thus, the higher domain area occupied by the microglia in E4FAD mice likely reflects swollen cell somas and hypertrophic processes, and not simply an increase in microglia density (Figure 11B). Collectively, these data suggest that APOE genotype differentially affects activation of microglia in response to Aβ deposits, and that morphological profiles of microglial reactivity can serve as a useful measure of Aβ-driven neuroinflammation.

The effects of APOE genotype on the increased microglial activation in E4FAD mice may be due to differences in the form of apoE or in the amount of apoE. Several studies have reported lower levels of total apoE in the brains of APOE4 knock-in mice (Riddell et al., 2008; Sullivan et al., 2011; Vitek et al., 2009). We previously measured brain apoE levels in EFAD mice using a three-step sequential protein extraction protocol (using TBS, TBS-Triton X-100 and formic acid) (Youmans et al., 2012b). Levels of apoE4 were selectively lower than apoE3 in the TBS-Triton X-100 fraction, suggesting that the lower levels of apoE4 may be due to the fact that the apoE is less lipidated. Lower levels of properly lipidated apoE could lead to less receptor binding and attenuated inhibition of microglial activation at Aβ plaques (Pocivavsek et al., 2009a; Pocivavsek et al., 2009b). We previously found that APOE4 and APOE4 mice have higher levels of LPS-induced neuroinflammation and impaired synaptic viability than APOE3
mice, suggesting that the loss of apoE contributes to increased neuroinflammation (Zhu et al., 2012). These data are consistent with in vitro and in vivo data showing that Aβ-independent neuroinflammation is higher with APOE4 than APOE3 (Colton et al., 2004; Lee et al., 2012; Vitek et al., 2009). For a review, see (Keene et al., 2011).

The type and amount of apoE also can affect glial responses to other forms of Aβ, in addition to extracellular deposits. Our previous data demonstrate that levels of soluble Aβ42 and soluble oAβ are higher in both the hippocampus and cortex of E4FAD mice compared to E3FAD mice (Younmans et al., 2012b). Analysis of EFAD mice suggested that reduced lipidation of apoE4 results in lower levels of the apoE4/Aβ complex, resulting in increased levels of soluble oAβ (Tai et al., 2013; Tai et al., 2014). However, dissecting the effect of APOE-genotype-modulated extracellular Aβ vs soluble oAβ on microglial activation in vivo is complex, as soluble Aβ and insoluble Aβ exist in a dynamic compartmentalization (Hong et al., 2011), and oAβ levels are higher in microenvironments surrounding amyloid plaques (Koffie et al., 2012). Thus, both forms of Aβ likely contribute to microglial activation. Collectively, these data support the hypothesis that lipid-poor apoE4 may result in a loss of function not only on Aβ clearance, but also on Aβ-independent and Aβ-driven neuroinflammation, both of which are a focus of our ongoing studies.

In summary, we have shown that APOE genotype differentially impacted Aβ-plaque-associated microglial activation in the brains of EFAD mice. APOE4 increased levels of IL-1β and negatively affected morphological profiles of microglial reactivity within cortical Aβ plaque domains. In addition, APOE genotype affected characteristics of Aβ plaques in the EFAD subiculum, supporting previously reported data describing Aβ deposition in this model. Our data
support the use of the EFAD transgenic mouse model for studies of Aβ-associated neuroinflammation, and demonstrate the need for APOE-targeted therapeutics in AD aimed at regulating neuroinflammation.
CHAPTER IV

EFFECTS OF APOE GENOTYPE ON SPATIAL LEARNING & MEMORY.

Figure 12. Golgi stained tissue section selected for Learning & Memory cover. Artistic interpretation of a representative Golgi stained section used in our studies. Dendritic morphology of layers II/III MEC neurons were assessed in young APOE TR mice, in addition to behavioral performance in spatial learning and memory tasks.

\[ This \ chapter \ is \ modified \ from \ Rodriguez \ GA, \ Burns \ MP, \ Weeber \ EJ, \ Rebeck \ GW. \ (2013). \ Young \ APOE4 \ targeted \ replacement \ mice \ exhibit \ poor \ spatial \ learning \ and \ memory, \ with \ reduced \ dendritic \ spine \ density \ in \ the \ medial \ entorhinal \ cortex. \ Learning \ & Memory. \ 20 \ (5):256-66. \ (PMID: \ 23592036) \]
A. Overview

Our understanding of how APOE genotype affects CNS pathological states has grown since its original association with AD was discovered (Corder et al., 1993; Strittmatter et al., 1993). To test the hypothesis that APOE genotype affects normal brain function in the absence of AD pathology, I assessed spatial learning and memory in human APOE mice using two different tasks: the Barnes maze and the HPWM. Chapter IV contains the results of these experiments in both young and old mice, along with an analysis of neuronal dendritic morphology in the MEC of young APOE mice. The MEC is an important source of cortical input to the hippocampus that conveys spatial information, and plays a critical role in spatial representation and navigation in rodents (van Groen et al., 2003; van Strien et al., 2009; Witter and Moser, 2006). Importantly, the EC is the first brain region to succumb to AD pathology in humans, and the neurons in the region analyzed may represent a particularly vulnerable cell population to pathology as the animal ages.
B. Experimental Results

1. Young APOE4 Mice Exhibit Impaired Learning & Memory

The Barnes Maze

We first trained three-month old APOE mice (E2, n = 12; E3, n = 10; E4, n = 13) of mixed sexes to locate a TH in a circular Barnes maze consisting of 20 holes evenly spaced along the perimeter (Barnes, 1979; Berta et al., 2007). Occupancy plots for all APOE mice on each TD were generated to qualitatively assess the location and duration of exploratory behavior in the maze (Figure 13). During TD1, mice from each APOE genotype actively explored all of the holes along the perimeter of the maze in order to locate the TH (arrow). By TD4, APOE2 and APOE3 mice spent little time investigating false holes, as indicated by the cooler colors appearing adjacent to the false holes (Figure 13). In contrast, occupancy plots for APOE4 mice on TD4 showed warmer colors surrounding the false holes, indicating a greater degree of time spent investigating other areas of the maze (Figure 13).
Figure 13. Location and duration of exploratory behavior in the Barnes maze. 3-month old APOE mice were trained over four TDs to locate a TH in the Barnes maze. To qualitatively assess and visualize the areas where our mice spent the most amount of time, we generated color-coded occupancy plots for each group of APOE mice and collapsed the trials by training day. On TD1, all mice spent an equal amount of time exploring the false holes. By TD4, the mice spent less time along the perimeter, suggestive of learning. However, APOE4 mice appeared to spend more time exploring false holes than APOE2 and APOE3 mice, as indicated by the warmer colors around false holes in the maze. Black arrow: TH. Color-coding: Blue, least time; Red, most time.
To quantify this behavior, we measured the latency of the mice to enter the TH and escape the maze. We found a significant effect of APOE genotype \( F(2,96) = 21.36, P < 0.001 \) and TD \( F(3,96) = 108.56, P < 0.001 \) on latency to TH, but no interaction between factors. Each group of APOE mice learned to locate the TH over the spatial acquisition phase, but post hoc analyses revealed that APOE4 mice required significantly more time than APOE3 animals to locate the TH and escape the maze at each TD (TD1, \( P < 0.05 \); TD2, \( P < 0.001 \); TD3, \( P < 0.001 \); TD4, \( P < 0.05 \)), suggestive of learning deficits in this spatial task (Figure 14A). Escape latency was not significantly different between APOE2 and APOE3 mice. In addition to measuring escape latency across training, we measured the total distance traveled as a conventional measure of behavioral performance on the Barnes maze. We also measured average speed and total number of nose pokes to assess locomotor activity and exploratory drive, respectively. We found significant main effects of APOE genotype \( F(2,96) = 9.02, P < 0.001 \) and TD \( F(3,96) = 63.88, P < 0.001 \) on total distance traveled, with no interaction between factors (Figure 14B). APOE4 mice traveled farther than APOE3 mice to the reach the TH on TD2 \( (P < 0.001) \) and TD3 \( (P < 0.01) \). We found a significant interaction between APOE genotype × TD on average speed \( F(6,96) = 3.03, P < 0.01 \), with significant main effects of both APOE genotype \( F(2,96) = 20.22, P < 0.001 \) and TD \( F(3,96) = 16.14, P < 0.001 \) (Figure 14C). Post hoc comparisons revealed no differences between APOE3 and APOE4 mice on the first three days of training. However, APOE4 mice were significantly slower on the maze than APOE3 mice on TD4 \( (P < 0.001) \). The average speed of APOE2 mice was also significantly slower than that of APOE3 mice on TD4 \( (P < 0.05) \). As for exploratory drive, APOE4 mice maintained a high level of nose poke investigations throughout training, while APOE2 and APOE3 mice showed decreases over time. Significant
main effects for APOE genotype ($F_{(2,96)} = 16.73, P < 0.001$) and TD ($F_{(3,96)} = 17.69, P < 0.001$) on total nose pokes were detected, with no interaction between factors (Figure 14D). Post hoc analyses revealed no differences in performance on TD1, although significant differences for APOE4 mice did appear on subsequent TDs (TD2, $P < 0.001$; TD3, $P < 0.01$; TD4, $P < 0.01$).

To determine the total number of errors made as the animals acquired the task, we subtracted the number of TH investigations from total nose pokes. Expression of total errors made during training did not differ considerably from our graphs showing total nose pokes; Figure 14D is thus doubly informative in that it provides evidence for increased errors in young APOE4 mice. Test performances in any of these measures were not significantly different between APOE2 and APOE3 animals.
Figure 14. Young APOE4 mice exhibit poor spatial learning in the Barnes maze. 3-month old APOE mice were trained over four TDs to locate a TH in the Barnes maze. (A) APOE4 mice exhibit increased latency to escape the maze over four TDs compared to APOE3 mice that cannot be attributed to differences in running speed (C). (B) APOE4 mice also traveled farther than APOE3 mice on TD3 and TD4. (D) Whereas exploratory activity did not differ by APOE genotype on TD1, APOE4 animals spent more time investigating false holes than APOE2 or APOE3 mice on subsequent TDs (see also Figure 15). Two-way ANOVA with repeated measures. Post hoc analyses, APOE4 vs APOE3, * P < 0.05, ** P < 0.01, *** P < 0.001. APOE2 vs APOE3, # P < 0.05.
To test whether our young APOE mice had established a spatial discrimination bias in the maze over training days, we divided the surface area of the Barnes maze into four equally sized zones containing five holes each, and measured time spent actively exploring the zones. We found that all groups increased the percent time exploring the target zone across TDs ($F_{(3,96)} = 18.70, P < 0.001$), with no effect of APOE genotype (Figure 15). To test whether our mice had comparable levels of behavioral activity upon exposure to the spatial acquisition phase, we examined escape latency across the first four training trials during TD1. We found significant main effects of APOE genotype ($F_{(2,96)} = 3.73, P < 0.05$) and trial ($F_{(3,96)} = 21.14, P < 0.001$) on latency to TH, indicative of increased performance even within the first TD. However, post hoc group differences were only detected on trial four between APOE4 vs APOE3 mice ($P < 0.05$) (Figure 16A). All mice had comparable latency measures on the first training trial and moved at the same speed on average (Figure 16B, C). To determine whether performance in the Barnes maze was influenced by APOE genotype in both males and females, we divided our APOE mice by sex and analyzed latency to enter the TH and escape the maze. Escape latencies were similarly affected by APOE genotype in both females ($F_{(2,39)} = 8.73, P < 0.01$) and males ($F_{(2,48)} = 17.61, P < 0.001$) ($E2$, $F = 6/M = 6$; $E3$, $F = 5/M = 5$; $E4$, $F = 5/M = 8$) (Figure 17).
Figure 15. Young APOE4 mice develop a spatial discrimination bias during training. 
Left: A standard Barnes maze for behavioral testing is shown. During the spatial acquisition phase of Barnes maze testing, the maze was divided into four zones of equal size surrounding the center of the maze. Each of the four zones contained five holes along the perimeter. The shaded zone depicts a representative target zone, which contained the TH (red arrow). Right: At 3-months of age, all APOE mice increased percent time spent in the zone containing the TH (target zone) over the four TDs.
Figure 16. **APOE genotype influences initial learning on the Barnes maze, but not baseline behavioral activity.** Behavioral performance on TD1 was analyzed to assess effects of APOE genotype on Barnes maze activity during the first day of training. (A) The scatter plot depicts the latency to enter the target hole and escape the maze for each individual APOE animal tested (APOE2 – white diamonds, APOE3 - gray diamonds, and APOE4 - black diamonds). If the animal had not entered the target hole by 180 sec, the trial was terminated. While APOE2 and APOE3 mice performed equally well during all training trials, APOE4 mice required significantly more time to locate and enter the TH at the fourth trial. (B) During the first trial alone, escape latency is not affected by APOE genotype or speed in the maze (C). Two-way ANOVA with repeated measures. Post hoc analyses, APOE4 vs APOE3, *P < 0.05.
Figure 17. Influence of sex on behavioral performance of young APOE mice in the Barnes maze. Behavioral performance in our APOE TR mice (E2, F = 6/M = 6; E3, F = 5/M = 5; E4, F = 5/M = 8) was analyzed by sex to determine whether APOE genotype influenced male or female spatial learning differently. At 3-months of age, APOE4 genotype was associated with increased escape latency in both our female and male mice. Two ANOVA with repeated measures. Post hoc analyses, APOE4 vs APOE3, * P < 0.05, ** P < 0.01. APOE2 vs APOE3, ## P < 0.01.
Twenty-four hours and seventy-two hours after the last training trial, the TH was closed and animals were tested in a single 90 sec probe trial to assess long-term spatial memory. During the 24-hr probe, we found a trend toward increased primary latency in young APOE4 mice, defined as the time required to initially investigate the closed TH ($F_{(2,31)} = 3.28$, $P = 0.058$) (Figure 18A). Primary latency in APOE4 mice (17.93 ± 2.90 sec) during the first probe trial was lower than the escape latency recorded during TD4 (43.85 ± 5.45 sec), indicative of improved performance between TD4 and the probe trial. No differences in primary errors, primary speed, or total nose pokes (Figure 18B-D) were detected between groups during the 24-hr probe trial. When we evaluated spatial preference for the closed TH and two adjacent holes, we found a significant effect of TH investigation in all APOE mice compared to non-THs on the opposite side of the maze ($F_{(1,62)} = 79.31$, $P < 0.001$) (Figure 19). Thus, all APOE mice had developed and expressed a spatial preference for the side of the maze containing the TH. At the 72-hr probe, we detected a difference in primary latency ($F_{(2,30)} = 4.90$, $P < 0.05$) between groups, with APOE4 mice requiring significantly more time to locate the TH than APOE3 mice (32.22 ± 6.55 sec vs 11.24 ± 5.22 sec; post hoc analysis, $P < 0.05$) (Figure 18E). Primary errors were also elevated in APOE4 mice (APOE genotype, $F_{(2,30)} = 6.70$, $P < 0.01$; post hoc analysis, $P < 0.05$) (Figure 18F). No differences in primary speed or total nose pokes (Figure 18G, H) were detected among APOE genotypes, and all mice expressed a preference for the closed TH ($F_{(1,58)} = 43.97$, $P < 0.001$) (Figure 19).
Figure 18. Spatial memory is impaired in young APOE4 mice 72-hr after training. During the probe trials, the TH was closed and all mice were given 90 sec to freely explore the maze. (A) Twenty-four hours after training, APOE4 mice showed a trend toward higher primary latency. (B-D) There was no effect of APOE genotype on primary errors, primary speed, or total nose pokes on the maze during this probe. (E-F) Seventy-two hours after training, APOE4 mice showed increased primary latency and more primary errors compared to APOE3 mice. (G-H) Primary speed and total nose pokes did not differ between APOE genotypes. One-way ANOVA. Post hoc analyses, APOE4 vs APOE3, * P < 0.05, ** P < 0.01.
Figure 19. Young APOE4 mice express a spatial discrimination bias during probe trials. During the 24- and 72-hr probes, total investigations of the closed TH and adjacent holes were averaged for each mouse. In addition, average investigations of the three opposite holes (non-target) were collected. Middle red arrow, closed TH. Black arrows, adjacent targets. Black Xs, non-THs. All 3-month old APOE mice expressed a spatial preference for the THs over the non-THs. Two-way ANOVA. Post hoc analyses, THs vs non-THs, * $P < 0.05$, *** $P < 0.001$. 
Hidden Platform Water Maze

The HPWM is another highly utilized behavioral test for assessing spatial learning and memory in rodents. We trained a comparable population of naïve young (3-month old) APOE mice (E2, n=10; E3, n=12; and E4, n=9) of mixed sexes to find a hidden platform over four training days (HDs) using a protocol similar to that of the Barnes maze. In addition, we tested our mice in the same behavioral suite and utilized the same distal, extra-maze visual cues.

Occupancy plots comparing patterns of activity in the HPWM on HD1 and HD4 qualitatively demonstrate greater spatial bias at the end of training for all APOE groups, indicative of learning behavior (Figure 20A). To quantify this, we measured the cumulative distance to platform and latency to reach the platform across HDs. We found a significant main effect of HD on cumulative distance to platform ($F_{(3,84)} = 64.30, P < 0.001$), but no effect of APOE genotype ($F_{(2,84)} = 2.06, P = 0.15$) (data not shown). When we consider latency to reach the platform as our dependent measure, we found a significant main effect of training day as well ($F_{(3,84)} = 76.70, P < 0.001$), with no effect of APOE genotype (Figure 20B). Post hoc analyses revealed that genotype influenced our dependent measure at HD2 only (APOE4 vs APOE3; $P < 0.05$). However, training day accounted for 61% of the variability in our model, while only 2% was due to APOE genotype. All groups reduced total distance traveled to platform over training days ($F_{(3,84)} = 64.62, P < 0.001$), with APOE2 mice traveling farther than APOE3 mice during the first two days (HD1, $P < 0.01$; HD2, $P < 0.01$) (Figure 20C). APOE2 swim speeds were significantly higher than APOE3 mice (HD1, $P < 0.01$; HD3, $P < 0.05$; HD4, $P < 0.01$) (Figure 20D). However, we did not detect differences in swim speeds or total distance traveled to platform between APOE3 and APOE4 mice over training.
Figure 20. Young APOE4 mice do not exhibit impaired spatial learning in the HPWM.
A naïve cohort of 3-month old APOE mice (E2, n=10; E3, n=12; and E4, n=9) were tested in the HPWM. (A) Occupancy plots comparing activity patterns on HD1 and HD4 in our APOE mice illustrate help visualize improved performance over training, as well as a hyperactive phenotype in our APOE2 mice. Arrows indicate target quadrant. (B) Time in seconds (latency to platform) to reach the hidden platform was measured over four HDs (HD 1-4). Behavioral performance in the hidden platform phase and during visible platform trials (VT) of the HPWM was not affected by APOE genotype. (C) Distance traveled during these sessions was also measured. APOE2 mice swam farther than APOE3 mice on the first two days of training, but performed similarly to APOE3 mice on subsequent days. (D) APOE2 mice swam faster on average than APOE3 mice over training. However, we did not detect differences in swim speeds between APOE3 and APOE4 mice. Two-way ANOVA with repeated measures. Post hoc analyses, APOE4 vs APOE3, * P < 0.05. APOE2 vs APOE3, # P < 0.05, ## P < 0.01.
No main effects of APOE genotype were found when analysis was restricted by sex (E2: F=4/M=6; E3: F=8/M=4; E4: F=4/M=5), though post hoc comparisons did detect a group difference in performance on HD2 in females ($P < 0.01$; APOE4 vs APOE3) (Figure 21).

**Figure 21. No influence of sex on performance of young APOE mice in the HPWM.** Behavioral performance in our APOE mice (E2: F = 4/M = 6; E3: F = 8/M = 4; E4: F = 4/M = 5) was analyzed by sex to determine whether APOE genotype influenced male or female spatial learning differently. No overall effects of APOE genotype was found in either sex, though female APOE4 mice took longer to find the platform than APOE3 females on HD2. Two-way ANOVA with repeated measures. Post hoc analyses, APOE4 vs APOE3, * $P < 0.05$. 

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Seventy-two hours after training, we removed the hidden platform and recorded behavior in a single 90 sec probe trial to assess long-term memory trace stability. APOE genotype did not affect the percentage of time spent in the Target Quadrant between groups ($F_{(2,29)} = 1.67, P = 0.21$), although within-group $t$-tests revealed above chance (25%) navigation in the Target Quadrant for APOE3 mice alone ($t_{(11)} = 2.96, P < 0.05$) (Figure 22A). When compared to percent time spent in other quadrants, only APOE3 mice spent significantly more time in the Target Quadrant (Target vs Other Quadrants, $P < 0.001$). We also counted the number of platform site crossings in the Target Quadrant as a more stringent measure of memory performance (Figure 22B), but did not detect differences in crossings or time spent swimming towards the platform site between groups. There was no effect of sex on any behavioral measures in the 72-hr probe (data not shown). To test the visual acuity of our young APOE mice, we ran three sequential trials (90 sec; ITI = 30 min) 1-hr after the 72-hr probe with the platform 2 cm above the water’s surface in a new quadrant each trial. Latency to reach the platform was shorter for all groups relative to HD4, with no genotype differences detected (Figure 20B).

Thus, unlike in the Barnes maze, behavioral performance deficits were not detected in APOE4 mice during the spatial acquisition phase of the HPWM. However, APOE4 performance in a long-term memory probe trial was affected.
Figure 22. Young APOE4 mice exhibit impaired spatial memory in the HPWM.
Seventy-two hours after training, the hidden platform was removed from the maze (SW quadrant) and behavior was assessed in a single 90 sec probe trial. (A) During the probe trial, we did not detect an effect of APOE genotype on time spent swimming in the target quadrant. Within-group analyses show that APOE3 mice alone spent more time swimming in the target quadrant than chance. (B) We did not detect APOE group differences in platform site crossings in the probe trial. One-way ANOVA, $\text{SSS } P < 0.001$, E3 (% time in target vs other quadrants). Within-group t-tests, $^+ P < 0.05$, E3 (% time spent in target quadrant vs 25%).
Open Field Test

Prior to Barnes maze and HPWM testing, we recorded behavior in an open field to identify any gross differences in locomotor activity or exploration that might interfere with initial habituation or training. We did not identify significant differences between APOE4 and APOE3 mice in any behavioral measures (Table 2). However, APOE2 mice did show significant differences in several locomotor and exploratory measures compared to APOE3 mice.

Together, these data show that 3-month old APOE4 mice exhibit performance deficits in the spatial acquisition phase and probe trials of the Barnes maze compared to APOE3 mice that are not due to differences in locomotor activity or exploratory drive.
Table 2. Locomotor and exploratory activity in young APOE mice in the OFT. (A) Behavior in a circular open field arena was recorded in young APOE mice (E2, n = 12; E3, n = 13; and E4, n = 17) prior to Barnes maze testing. The arena was divided into an inner zone and an outer zone. (B) Occupancy plots from a smaller cohort of APOE mice were generated to visualize activity in the open field. In general, APOE mice display thigmotaxis in the arena, with no overt differences in activity between APOE genotype. Table: Locomotor activity, exploration, and % time within the outer zone were elevated in young APOE2 mice. Total duration of rearing behavior was also elevated in APOE2 mice. There were no differences detected between APOE3 and APOE4 mice in any measure. One-way ANOVA, * P < 0.05, ** P < 0.01, *** P < 0.001.

<table>
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<th>APOE4</th>
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<td>Time in inner zone (%)</td>
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<td>Time in outer zone (%)</td>
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<td>Total distance traveled (m)</td>
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<td>Average speed (cm/s)</td>
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<td>Rearing (s)</td>
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2. Older APOE4 Mice Exhibit Learning Deficits

To determine whether behavioral performance deficits seen in young APOE4 mice on the Barnes maze persist or are exacerbated later in life, we trained a naïve, eighteen-month old cohort of APOE mice (APOE2, \( n = 8 \); APOE3, \( n = 10 \); and APOE4, \( n = 9 \)) to locate a randomly assigned TH using identical test procedures. Prior to habituation, we again assessed locomotor activity and exploratory drive in a circular open field arena (Table 3). All groups spent most of the time exploring the outer zone of the maze (86.88 ± 1.40%), with no differences among APOE genotypes. Likewise, no differences were detected in total distance traveled, average speed, or grooming behavior during 300 sec of free exploration. Duration of rearing behavior was significantly elevated in APOE2 mice \( (F_{2,24} = 14.87, \ P < 0.001) \); post hoc analysis, \( P < 0.01 \)), but not different between APOE3 and APOE4 animals.
Table 3. Locomotor and exploratory activity in older APOE TR mice in the OFT.
Behavior in a circular open field arena was recorded in 18-month old APOE mice (E2, \( n = 8 \); E3, \( n = 10 \); and E4, \( n = 9 \)) prior to Barnes maze testing. There were no group differences in general locomotor activity, exploration, and % time within the inner or outer zones of the open field. We did find that older APOE2 mice had an increased total duration of rearing behavior compared to APOE3 mice. One-way ANOVA, *** \( P < 0.001 \).
We carried our 18-month old APOE mice through the Barnes maze habituation and spatial acquisition phases following OFT. As with the younger cohort of animals, we found significant main effects of APOE genotype \( (F_{(2,72)} = 4.83, P < 0.05) \) and TD \( (F_{(3,72)} = 23.16, P < 0.001) \) on total distance traveled, with APOE4 mice traveling farther than APOE3 mice to reach the TH (data not shown). Occupancy plots showed that APOE mice spent less time overall exploring the maze by TD4 (Figure 23A). When we quantified the latency to enter the TH and escape the maze, we found significant main effects of APOE genotype \( (F_{(2,72)} = 7.01, P < 0.01) \) and TD \( (F_{(3,72)} = 58.38, P < 0.001) \), but no interaction between factors (Figure 23B). All groups required over 90 sec on TD1 to locate and enter the TH, with no differences in performance between APOE3 and APOE4 mice. By TD4, APOE3 mice required 18.6 ± 2.0 sec to escape the maze, whereas APOE4 mice required over twice that amount, although this difference was not statistically significant \( (P > 0.05) \). Post hoc analyses revealed that APOE4 mice required significantly more time to escape the maze than APOE3 mice on TD2 only \( (P < 0.05) \). In addition to escape latency, we measured the average speed of our mice during training, as well as the total number of nose pokes in the maze. Average speed in older mice was similar to that of our younger cohort (Figure 14C). We found significant main effects of APOE genotype \( (F_{(2,72)} = 4.01, P < 0.05) \) and TD \( (F_{(3,72)} = 34.68, P < 0.001) \) on average speed, with post hoc analyses showing that APOE3 mice perform faster than APOE4’s on TD3 \( (P < 0.01) \) and TD4 \( (P < 0.001) \) (Figure 23C). Finally, total nose pokes and total errors made over training were similar to that of our younger cohort (data not shown). Significant main effects were detected for APOE genotype \( (F_{(2,72)} = 5.19, P < 0.05) \) and TD \( (F_{(3,72)} = 7.01, P < 0.001) \) on total nose pokes, with no interaction between factors. Test performance was not significantly different between 18-month
old APOE2 and APOE3 mice. When the maze was split into four equal zones, we found that all APOE mice expressed a similar spatial preference for the Target Zone over TDs ($F_{(3,72)} = 19.24$, $P < 0.001$) (Figure 23D).

**Figure 23.** Impaired spatial learning on the Barnes maze in an older cohort of APOE4 mice. Naïve 18-month old APOE mice (E2, $n = 8$; E3, $n = 10$; and E4, $n = 9$) were trained on the Barnes maze to assess cognitive function in old age. (A) Occupancy plots for a smaller cohort of old APOE mice show preference for the region of the maze containing the TH (black arrow) by TD4. (B) APOE4 mice required more time to locate the TH and escape the maze than APOE3 mice on TD2. (C, D) Differences in running speeds between APOE genotype were only detected on TD3 and TD4, reflecting a similar pattern of locomotor activity in our younger cohort. Additionally, all APOE groups developed a spatial bias in the Barnes maze.
Due to an inconsistent number of females vs males in our 18-month old APOE groups, we pooled APOE2 and APOE3 mice as non-APOE4 carriers (non-APOE4: F= 7/M= 11; E4: F= 4/M= 5) and compared their behavior to APOE4 mice. We were able to detect an effect of APOE genotype on latency to escape the maze in males ($F_{(1,42)} = 12.61, P < 0.01$), but not in females ($P > 0.05$) (Figure 24).
Figure 24. Influence of sex on behavioral performance of older APOE mice in the Barnes maze. Behavioral performance in our 18-month old cohort of APOE mice (non-APOE4: F= 7/M= 11; E4: F= 4/M= 5) was analyzed by sex to determine whether APOE genotype influenced male or female spatial learning differently. Due to an inconsistent number of females vs males in our older mice, APOE2 and APOE3 females and males were grouped as non-APOE4 carriers and compared to APOE4 females or males. Increased latency to escape the maze was only found in male APOE4 mice on TD1 and TD2. Two ANOVA with repeated measures. Post hoc analyses, APOE4 vs APOE3 males, $^s P < 0.05.$
Twenty-four and seventy-two hours after the last training trial, spatial LTM was probed in individual 90 sec trials with the TH closed. No genotype differences were detected in primary latency during the 24-hr probe \((F_{(2,23)} = 1.30, P > 0.05)\) (Figure 25A). Interestingly, the difference in APOE4 escape latency at TD4 \((47.1 \pm 5.8 \text{ sec})\) vs APOE3 escape latency \((18.6 \pm 2.0 \text{ sec})\) was reduced only 24-hr later \((\text{APOE3, } 19.5 \pm 3.6 \text{ sec}; \text{APOE4, } 18.7 \pm 2.4 \text{ sec})\). When we examined primary errors, we found no differences between groups \((F_{(2,23)} = 3.36, P = 0.053)\) (Figure 25B). However, we did detect genotype differences in primary speed \((F_{(2,23)} = 3.96, P < 0.05)\) and total nose pokes \((F_{(2,24)} = 6.96, P < 0.01)\) (data not shown), with APOE4 mice performing the slowest and investigating the fewest holes total compared to APOE2 \((P < 0.05)\) or APOE3 mice \((P < 0.01)\), respectively. When we assessed behavioral performance 72-hr after training, we detected an effect of APOE genotype on primary latency \((F_{(2,21)} = 5.58, P < 0.01)\) (Figure 25C), although this was restricted to APOE4 vs APOE2 animals \((P < 0.01)\). APOE3 mice had a primary latency of 19.5 \pm 3.1 sec, whereas APOE4 mice required 27.9 \pm 2.8 sec to investigate the closed TH. We did not detect group differences in primary errors, primary speed, or total nose pokes in the 72-hr probe trial.

Thus, within a naïve cohort of 18-month old APOE mice, we were able to detect a similar pattern of activity in the spatial acquisition phase of the Barnes maze to that in our 3-month old APOE mice.
Figure 25. **Spatial LTM in older APOE mice in the Barnes maze.** Twenty-four and seventy-two hours after training, the TH was closed and behavioral performance was assessed in single 90 sec probe trials. **(A-B)** Primary latency and primary errors were not effected by APOE genotype 24-hr after training. **(C-D)** During the 72-hr probe, older APOE4 mice exhibited increased primary latency compared to APOE2 mice, but not APOE3 mice. Primary errors were not influenced by APOE genotype in this probe trial. One-way ANOVA.
3. APOE4 Alters Dendritic Morphology of MEC Neurons

Based on our behavioral results with young APOE mice, we were interested in determining whether APOE genotype differentially affected dendritic morphology in MEC principal cells. We analyzed dendrites in a cohort of 3-month-old female APOE mice (E2, \( n = 3 \); E3, \( n = 4 \); E4, \( n = 4 \)) that had not undergone behavioral testing by processing their brains for Golgi-Cox impregnation (Figure 26A). Pyramidal cells within MEC layers II/III (E2, \( n = 8 \); E3, \( n = 6 \); E4, \( n = 10 \)) were traced, coded, and analyzed by an investigator blinded to APOE genotype. APOE genotype had a significant effect on total dendritic length of these cells (\( F_{(2,21)} = 3.66, \ P < 0.05 \)) (Figure 26B, left): total length of MEC pyramidal cell dendrites was 33% shorter in APOE4 cells compared to APOE3 cells (\( P < 0.05 \)). No significant difference in total dendritic length was observed between cells in APOE2 and APOE3 brains. We found significant main effects of APOE genotype (\( F_{(2,42)} = 3.52, \ P < 0.05 \) and dendritic compartment (\( F_{(1,42)} = 4.28, \ P < 0.05 \)) on the length of MEC pyramidal cell dendrites, with post hoc analyses revealing significantly shorter basal shaft dendrites in APOE2 (\( P < 0.05 \)) and APOE4 (\( P < 0.001 \)) MEC neurons compared to APOE3 neurons. In contrast, APOE genotype did not affect the length of apical dendrites (Figure 26B, right). No differences in node (branch) number or number of primary dendrites leaving the cell soma were detected (data not shown).
Figure 26. Dendritic length of layers II/III MEC pyramidal cells is reduced in young APOE4 mice. Basal dendrites, but not apical or apical oblique, are affected. Golgi-Cox staining was performed on brains from 3-month old APOE mice. Dendritic length of MEC pyramidal cells were measured from sections of APOE mouse brains (APOE2, n = 3 mice/8 cells; APOE2, n = 4/6; APOE4, n = 4/10) (A) Left: Brains were sectioned sagittally to visualize cells in the MEC. Scale bar, 500µm. Center: Rotated image of the MEC with dorso-ventral axis shown on the left. Scale bar, 200µm. Right: Reconstructed image of a representative MEC pyramidal cell with traced dendrites (cyan). Scale bar, 30µm. (B) Total dendritic length in APOE4 neurons was significantly reduced relative to APOE3 neurons. Basal dendrites were found to be significantly shorter in APOE2 and APOE4 cells compared to those in APOE3 cells. One-way or two-way ANOVA. Post hoc analyses, APOE2 or APOE4 vs APOE3, * P < 0.05, *** P < 0.001.
In addition to dendritic length, we analyzed the dendritic spine density of layers II/III MEC pyramidal cells imaged (Figure 27). All spine density analysis was performed by an investigator blinded to APOE genotype. We found a significant effect of APOE genotype on dendritic spine density ($F_{(2,18)} = 7.01, P < 0.01$), with APOE4 cells possessing 18% fewer spines/10μm of dendritic segment compared to APOE3 cells ($P < 0.05$) (Figure 27). Spine densities did not differ between APOE2 and APOE3 cells. We also compared spine densities within apical dendrites and basal shaft dendrites. We found significant main effects of APOE genotype ($F_{(2,36)} = 8.25, P < 0.01$) and dendritic compartment ($F_{(1,36)} = 8.27, P < 0.01$), with a significant interaction between factors ($F_{(2,36)} = 3.78, P < 0.05$). Post hoc analyses revealed that APOE4 dendritic spine density was significantly (34%) lower in basal shaft dendrites compared to APOE3 spine density in the same compartment ($P < 0.001$) (Figure 27). Interestingly, we did not detect APOE genotype differences in dendritic spine density in apical dendrites.
Figure 27. Dendritic spine density of layers II/III MEC pyramidal cells is reduced in young APOE4 mice. Basal dendrites, but not apical or apical oblique, are affected. (A) Representative photomicrographs of basal dendritic segments for each APOE genotype are shown at 63X magnification. A heterogeneous population of dendritic spines were present in all cells imaged. (B) Left: Total dendritic spine density of MEC neurons was lower in APOE4 mice compared to APOE3 mice. Right: When analyzed as a function of dendritic compartment, APOE4 basal dendrites alone exhibited reduced spine density. One-way or two-way ANOVA. Post hoc analyses, APOE4 vs APOE3, * $P < 0.05$, ** $P < 0.001$. 
C. Discussion

In this series of experiments, we found that young (3-month old) APOE4 mice had spatial learning and memory deficits as determined in the Barnes maze, and that APOE4 genotype was associated with reductions in MEC dendritic length and spine density at this age. Impaired spatial learning in APOE4 animals was also detected in an independent older (18-month old) cohort of mice, demonstrating a continuous, negative effect of APOE4 genotype on behavioral performance into late adulthood. These data support the idea that APOE genotype differentially modulates cognitive function throughout the lifespan, with APOE4 already affecting cognitive processes early in life.

Deficits in spatial learning and memory due to E4 have mainly been reported in older mice (Andrews-Zwilling et al., 2010; Bour et al., 2008), although some reports demonstrate impaired spatial recognition and contextual fear conditioning in younger animals (Grootendorst et al., 2005; Segev et al., 2013). To probe spatial cognition in young APOE mice, we used two behavioral paradigms: the Barnes maze and the HPWM. The Barnes maze is a dry-land maze well suited for assessing spatial learning and memory in mice (Mayford et al., 1996; O'Leary and Brown, 2012). Similar to the water maze, mice are trained to use visuo-spatial cues in the local or extra-maze environment to orient themselves and find a specific target. The task is typically acquired over several training trials and can be easily modified to suit the experimental needs of the investigator (Bour et al., 2008; Koopmans et al., 2003; O'Leary and Brown, 2012). We found that young APOE4 mice exhibited a strong deficit in behavioral performance during the spatial acquisition phase, suggestive of impaired spatial learning (Figures 13 & 14). This behavioral deficit in APOE4 mice was not due to differences in locomotor activity across TDs, as post hoc
analyses show that running speeds differed between APOE4 and APOE3 mice on TD4 only (Figure 14C). Likewise, APOE4 behavioral deficits were not due to decreased exploratory drive, as total nose pokes remained elevated in APOE4 mice relative to APOE3 mice during training (Figure 14D). In our older cohort of mice, we were able to detect a similar pattern of behavioral performance during spatial acquisition (Figure 23). APOE genotype influenced latency to locate the TH and escape the maze, with post hoc tests revealing significantly worse performance in APOE4 mice on TD2 compared to APOE3. Performance differences between these groups on the two remaining TDs approach significance under post hoc analyses. The pattern of locomotor activity (Figure 23A-C) and exploration across training was similar in young and old APOE mice, suggesting use of a spatial strategy to find the TH. To quantitatively express this strategy, we evaluated percent time spent in the target zone in young and old APOE mice, which showed expression of a spatial bias over training (Figures 15 & 23D). Importantly, occupancy plots generated on TD4 for young APOE4 mice did not show preferences for other regions of the maze or for specific holes other than the TH, lending support for the establishment of a goal-specific spatial discrimination bias (Figure 13). Finally, to determine whether poor baseline performance and locomotor activity in young APOE4 mice played a role in poor overall performance on the Barnes maze, we analyzed escape latencies and average speeds of APOE mice over the first four training trials (TD1). Trial 1 escape latencies and running speeds were not affected by APOE genotype, suggesting that all mice had similar baseline activity upon exposure to Barnes maze training (Figure 16). Taken together, our Barnes maze data showing behavioral performance deficits in APOE4 mice provide evidence for APOE4-induced learning impairment early in life.
We sought to minimize or eliminate the influence of non-mnemonic factors in our behavioral tasks. Nonetheless, several factors unassociated with spatial learning processes may have influenced behavioral performance in our mice. For instance, heightened levels of anxiety or fear in our APOE4 mice may have resulted in increased escape latency in Barnes maze training. Though we did not measure anxiety levels in our mice directly, we habituated our experimental animals to testing and handling procedures prior to administration of the Barnes maze in order to reduce stress. We also cleaned the maze thoroughly after each experimental trial to help eliminate odor trails left by previously tested mice. Occupancy plots did not detect APOE4-specific freezing behavior upon initial exposure to the Barnes maze (TD1) or at the end of training (TD4) (Figure 13). We also did not detect genotype differences in latency to exit the center of the maze (data not shown), a potential measure of stress reactivity. Importantly, decreased motivation and/or attention in APOE4 mice may have also played a role in Barnes maze performance deficits. Bright overhead lighting and weak aversive auditory stimulus (WAAS) were used to drive escape motivation in APOE mice in place of deprivation procedures (Koopmans et al., 2003). These stimuli may have been perceived by APOE4 mice differently upon initial exposure to the maze and/or over TDs. We hypothesize that a lack of motivation or attentiveness to spatial cues would interfere with baseline activity or spatial discrimination in the maze and confound our other dependent measures. However, escape latencies and speed measured on the first trial of TD1 did not reveal group differences in baseline locomotor activity (Figure 16). In addition, we did not detect genotype differences in the development of a spatial bias during training in either young or old APOE mice (Figures 15 & 23D).
Probe trials assessing long-term spatial memory in our young and old mice were conducted 24-hr and 72-hr after successful Barnes maze training. Importantly, all young APOE mice showed a clear preference for investigating the closed TH and two adjacent holes compared to non-THs on the opposite side of the maze (Figure 19). 18-month old APOE3 and APOE4 mice showed a similar spatial preference (data not shown). During the 24-hr probe, no effect of APOE genotype on two indicators of spatial memory, primary latency and primary errors, was detected in either age group (Figures 18 & 25). Performance on these measures may reflect treatment of the 24-hr probe as the first trial of a “fifth” TD, suggesting that, in spite of the performance deficit exhibited earlier during training, APOE4 mice eventually learn the task and perform comparably to APOE3 mice. When we examined behavior in the 72-hr probe, we found significant performance deficits in young APOE4 mice on primary latency and primary errors (Figures 18). This increase in errors and time required to locate the closed TH could be due to several causes. One possibility is that successive probe testing in our animals may have led to enhanced extinction learning in young APOE4 mice. However, we would not expect that a single 90 sec probe trial would induce extinction of the original spatial memory trace, as the spatial location of the TH was established in 16 training trials spanning four days. Moreover, primary latency and primary error measurements in the 72-hr probe did not change in young APOE2 and APOE3 mice. A second possible cause underlying decreased performance in APOE4 mice in the 72-hr probe may be a change in motivation or attention from the previous probe trial. Such changes may lead to differences in performance between probe trials and APOE genotype, while not necessarily reflecting impaired spatial memory. A third possibility is that APOE4 mice were least able to recall the TH location compared to other APOE genotypes.
Using the HPWM, we did not detect overt genotype differences in spatial learning in young APOE mice (Figure 20). Importantly, the Barnes maze differs from the HPWM in that mice are allowed to naturally traverse the open field and freely explore the maze in order to locate the TH. Thus, performance on the Barnes maze reflects a balance between two innate drives in addition to learning and memory processes: a natural tendency to avoid open spaces set against an active drive to freely explore a novel environment. In contrast, the HPWM requires mice to constantly navigate a large pool in search of a platform and is not suitable for active exploration. Moreover, repeated training trials in the HPWM are more stressful and physically taxing to the animal than on the Barnes maze (Aguilar-Valles et al., 2005; Harrison et al., 2009). We hypothesize that, in the absence of free exploration, an elevated stress response in the water maze may facilitate spatial learning in young APOE4 mice and mask adverse effects of apoE4 on spatial cognition. In line with this hypothesis, acute elevations in stress-induced corticotrophin-releasing hormone have been shown to enhance excitatory transmission and hippocampal-dependent memory (Chen et al., 2012). APOE genotype had no overall effect on spatial acquisition or probe trial performance in HPWM, though within-group analyses show APOE3 mice perform well in the probe (Figures 20 & 22A). APOE4 mice did not demonstrate a spatial preference for the target quadrant, suggesting a deficit in retention memory. These data potentially reflect a masking influence of HPWM-induced stress activation on cognitive processing, resulting in similar behavioral performance during hidden platform training, but not in probe trial performance. In a recent study, 6- to 8-month old APOE4 mice exhibited enhanced spatial learning in the water maze when considering cumulative distance to (Siegel et al., 2012). Interestingly, measures of anxiety were also elevated in APOE4 animals compared to age-
matched APOE2 and APOE3 mice, supporting a role for stress in behavioral performance. Normal aging may counteract stress-induced facilitation of cognitive processing in APOE4 mice trained in the water maze, making phenotypic differences easier to detect in older mice (Andrews-Zwilling et al., 2010; Bour et al., 2008). Deficits in spatial acquisition were clearly present in both young and old APOE4 mice in the Barnes maze (Figures 13, 14, & 23), which may reflect a more natural expression of spatial acquisition and memory processes that is sensitive to subtle deficits in cognitive function. It is important to note that despite impaired spatial learning in APOE4 mice, all animals were able to acquire the task over the course of four TDs. We conclude that APOE4 has a subtle, but negative, impact on spatial cognition in human APOE mice that can be detected very early in adulthood. These results support the utility of the Barnes maze as an attractive assay to screen therapeutics aimed at targeting ApoE or its receptors, without the need to treat and test older mice.

Behavior of APOE mice recorded in the open field satisfied a key objective relevant to our Barnes maze study: to determine if baseline differences in locomotor or exploratory activity would interfere with habituation or training. Open field testing also exposed APOE mice to handling procedures, which may have helped reduce anxiety in subsequent behavioral testing. No differences in locomotor activity or exploration were detected between APOE3 and APOE4 mice at 3- or 18-month of age (Tables 2 & 3). In contrast, Siegel et al (2012) found that young and old APOE4 mice exhibit reduced exploration in an open field compared to age-matched APOE2 and APOE3 mice. This discrepancy may be due to the shorter duration of recording per test session in our study (5 min compared to 10 min), which could have allowed for greater bouts of inactivity in APOE4 mice following comparable amounts of active exploration in the first 5
min of the task. Interestingly, our young APOE2 mice showed increased performance in all behaviors measured in the open field besides grooming, though this hyperactive phenotype did not manifest during Barnes maze testing. We did detect elevated swim speeds and distance traveled in young APOE2 mice in the HPWM, making probe trial performance difficult to interpret for the APOE2 mice.

We chose to investigate dendritic morphology in female APOE mice based on the demonstrated sensitivity of cognitive performance in females to the APOE-ε4 allele (Andrews-Zwilling et al., 2010; Bour et al., 2008; Raber et al., 2000; Siegel et al., 2012). Compared to APOE3 mice, APOE4 mice showed lower dendritic spine densities of layers II/III pyramidal cells in the MEC and shorter overall length of dendritic arbors at 3-months of age (Figures 26 & 27). This observation is of particular relevance to our behavioral studies, as the EC is a key relay structure between the hippocampus and a variety of subcortical regions and association areas of cortex that convey multimodal and highly processed unimodal information (Canto et al., 2008). In humans, the EC (Brodmann area 28) is a large structure in the ventromedial temporal lobe that sends extensive projections to the hippocampal formation via the perforant pathway (Van Hoesen, 1997). It is one of the first structures to exhibit neurofibrillary tangles in AD, and exhibits massive neuronal loss in superficial layers as the disease progresses (Braak and Braak, 1995; Gomez-Isla et al., 1996; Van Hoesen et al., 1991). Interestingly, EC volume and subregional thickness are reduced in cognitively normal APOE-ε4 carriers (Burggren et al., 2008; Donix et al., 2010; Shaw et al., 2007), suggesting an early manifestation of cortico-hippocampal dysfunction in prodromal AD. Analyses of Golgi-stained MEC neurons by dendritic compartment reveal that reductions in total dendritic length and spine density of APOE4 neurons
were due to differences in basal shaft dendrites, but not apical or apical oblique dendrites (Figures 26B & 27B). These differences may be functionally associated with our behavioral studies using the Barnes maze, as basal shaft dendrites of MEC layers II/III cells likely receive intrinsic collateral innervation from MEC superficial and deep layer neurons (Canto and Witter, 2012; Quilichini et al., 2010). Reduced dendritic length and spine densities of basal shaft dendrites, but not apical dendrites, may reflect impaired local processing of spatial information within the MEC that disrupts spatial learning and memory. Apical dendrites of MEC layers II/III cells extend toward the pial surface and branch into tufts within layer I and superficial layer II, where they receive dense innervation from structures conveying sensory information: i.e., olfactory cortex, pre-subiculum, and postrhinal cortex (Burwell and Amaral, 1998; Canto and Witter, 2012). Visual testing revealed no group differences in young APOE mice (Figure 20B), suggesting that sensory input into the MEC is not disrupted in these mice and does not underlie impaired behavioral performances. Further studies using APOE mice could be done to determine whether APOE4 genotype affects firing properties of layers II/III principal cells in addition to altering dendritic morphology.

In summary, we detected APOE4-induced deficits in spatial cognition using the Barnes maze in two independent cohorts of animals at different ages. Furthermore, we found impaired dendritic morphology in APOE4 neurons in the MEC. Impaired spatial learning and memory in APOE4 mice likely reflect functional changes to neuronal networks subserving spatial information processing. These data suggest that ApoE isoforms affect normal MEC structure and normal brain functions of learning and memory early in life, which in later life may contribute to AD-related risk.
CHAPTER V
EFFECTS OF LXR ACTIVATION ON NEURONAL NETWORK ACTIVITY

Figure 28. Hippocampal neurons and glial cells form complex networks in vitro. Dissociated hippocampal cultures were plated at high density (~700 cells/mm²) and maintained for two to three weeks in vitro for our experiments. In this image, hippocampal neurons (red, MAP2) and astrocytes (green, GFAP) form complex cellular networks that allow multi-site extracellular recordings in the multielectrode array. Cell nuclei (DAPI) are labeled in blue. 10X magnification, DIV18. Scale bar: 30μm.
A. Overview

My behavioral results in the Barnes maze (Chapter IV) suggested that signaling mechanisms in circuits underlying spatial learning and memory functioned less effectively in the APOE4 mice. This led to the overarching hypothesis that APOE-ε4 predisposes individuals to AD-related cognitive decline early in life by negatively affecting network activity of neuronal populations in the brain. However, our basic understanding of APOE and its role in network signaling is poor. Thus, the experiments described in Chapter V were designed to investigate the role of LXR activation – which increases apoE levels - on network activity and bursting dynamics in a reduced preparation. I recorded extracellular activity from primary hippocampal neurons cultured in multielectrode arrays, and tested whether increased LXR activation would impact network signaling. These preliminary experiments in the MEA represent a new approach to studying APOE and its effects on network signaling, and provide new data that may help bridge the information gap between single cells and cognitive behavior.
B. Experimental Results

1. TO901317 Suppresses Bursting Activity

*Development of the Hippocampal Network*

To test the hypothesis that apoE influences the network activity of neuronal populations, we assessed the formation and functional properties of cultured hippocampal cells *in vitro* using immunostaining and MEA analyses. Rat primary hippocampal cells were plated at high density (~700 cells/mm$^2$) and maintained for approximately two weeks before LXR treatment. Cells adhere to the silicon nitride substrate of the MEA within 24hr after plating, and begin to extend visible processes directed towards adjacent cells. At DIV5, hippocampal neurons are readily visualized over the electrode grid of the MEA (*Figure 29A*), and neurites appear branched and healthy. In parallel experiments, dissociated hippocampal cells were plated onto glass coverslips at equal density and processed for ICC to determine structural maturation of the networks. Hippocampal neurons formed densely interconnected circuits at DIV18 (*Figure 29B*). In addition, a dense population of astrocytes were present and entwined within the neuronal networks.
Figure 29. Development of mature hippocampal networks in vitro. (A) The MEA has 59 surface electrodes arranged on an 8x8 grid in the center of the recording chamber (active zone). Hippocampal neurons plated in the MEA send processes out along the surface and make contact with adjacent cells to form dense cellular networks. Photomicrograph shows hippocampal neurons in the active zone at DIV5. Scale bar: 200µm. (B) In parallel experiments, we plated dissociated hippocampal cells onto glass coverslips. Neurons (MAP2, green) and astrocytes (GFAP, red) comprise the majority of cell types in our mature hippocampal networks. DAPI is shown in blue. By DIV18, dense neurites and astrocytic processes were detected in the networks. Scale bar: 20µm.
**Effects of TO901317 on ApoE Levels in Hippocampal Cultures**

Baseline spontaneous activity was recorded from our hippocampal cultures at DIV15 for 15 min according to previous reports (Clifford et al., 2011; Niedringhaus et al., 2013; Niedringhaus et al., 2012). Immediately following the recording session, each MEA was transferred to a sterile biosafety cabinet and randomly assigned to the following groups and treated accordingly: DMSO (n=3), 1µM TO901317 (n=3), or 5µM TO901317 (n=4). Cultures were maintained for five days at 37°C before a second 15 min recording session was conducted (DIV20).

LXR activation upregulates the expression of a number of target genes involved in cholesterol transport, including *Apoe* (Sodhi and Singh, 2013). CM from each MEA was collected and pooled for each group to determine levels of secreted apoE. In preliminary experiments, we detected increased apoE in CM of TO-treated cultures, with the largest increase in the 5µM TO-treated MEAs (*Figure 30*). After the drug was removed via media exchange, apoE levels were again probed from CM at DIV25 to evaluate any enduring effects of LXR activation. In these experiments, no difference in apoE levels were detected between DMSO-treated and CTRL non-treated cultures.
Figure 30. LXR activation increased ApoE levels in the MEAs. Pooled CM from each treatment group was electrophoresed on a 10% gel, transferred to a nitrocellulose membrane, and probed for apoE. Five days of 5µM TO901317 treatment (lane 5) increased levels of secreted apoE in the CM relative to DMSO (lane 3). ApoE levels were not different in CTRL non-treated (lane 2) and DMSO-treated (lane 3) cultures. Remaining drug was removed from cultures via media exchange and apoE levels were probed at DIV25; CM samples appear in the same order (lanes 7-10). Equal volumes for each experimental sample (30µL) were loaded into the gel wells, as shown in the Ponceau stain. 5µL of CM from rat primary glial culture were run in the 12th lane, and serve as a positive control.
Analysis of LXR Activation on Network Activity

Representative screen shots of spontaneous, high-pass filtered recordings in the MEA are shown (Figure 31). Each box within an MEA corresponds to 2 seconds of activity from one electrode. Each electrode detects electrical activity from neurons in the vicinity and a majority of the electrodes reveal robust activity (Niedringhaus et al., 2013). At 15 days in vitro, individual spikes and high frequency spiking activity (bursts) are detected. Representative screen shots of spontaneous activity after five days of drug treatment are also shown (Figure 31). To visualize network activity from our MEAs across all electrode channels and over a longer temporal scale, we created raster plots of spiking activity over a 180 sec time window. Representative raster plots for sister MEAs shown in the previous figure are displayed (Figure 32). Each row in a panel corresponds to an individual electrode in the MEA, and one vertical tick mark represents a spike detected by that electrode. By DIV20, DMSO-treated hippocampal networks exhibit organized bursting activity at a high frequency relative to baseline, and are followed by varying intervals of quiescence. These observations suggest that hippocampal networks organized spiking patterns into epochs of synchronous activity over the five day vehicle treatment. In contrast, 1µM TO- and 5µM TO-treated networks did not appear to exhibit this change in bursting activity. TO-treated hippocampal networks appeared to exhibit a decreased number of bursts relative to DMSO, while the total spikes detected within the 180 sec time window appear less organized than in control networks.
Figure 31. Spontaneous activity in hippocampal networks before and after LXR activation. Screenshots of spontaneous, high-pass filtered recordings in the MEA. Left, The network activity from three sister MEAs are shown at baseline (DIV15). Right, Spontaneous activity after five days of DMSO (n=3), 1µM TO (n=3), or 5µM TO (n=4) treatment (DIV20). Each box represents two seconds (x-axis) of recording from one electrode and the voltage scale (y-axis) ranges from ± 100mV.
Figure 32. Network spiking activity is altered after LXR activation. Left, Raster plots show 180 sec of spontaneous activity at 15 days in vitro from untreated hippocampal networks. Right, DMSO treated networks display an increase in organized bursting activity over five days, while 1µM TO and 5µM TO treated networks do not. One row in each panel corresponds to one electrode and one vertical tick mark represents a detected spike. Y-axis, Channel #1-59.
We calculated the intervals between spikes recorded in the MEA to assist in burst identification. Bursts were defined as periods of high frequency spiking activity that contained no less than 4 spikes with a maximum inter-spike interval (ISI) of 100 ms. The maximum ISI criteria for bursts was determined by examining frequency plots of ISIs in the recording sessions. Representative ISI frequency counts for each treatment group are plotted in log scale to visualize their distribution across a large timescale (1ms to 100 sec) (Figure 3). At DIV15, the first peak is clustered around shorter ISIs, which likely define the intervals between spikes in bursts. A second peak typically appears clustered between 1 and 10 sec, and likely reflects intervals between bursts (Niedringhaus et al., 2013). DMSO-treated hippocampal networks exhibited an increase in the peak clustered at shorter ISIs. In contrast, TO-treated hippocampal networks exhibited a decrease in the frequency counts of shorter ISIs.
Figure 33. Log histograms of inter-spike intervals (ISI) illustrate an effect of TO901317 on short ISI frequency. ISIs were calculated in seconds and their frequency counts were plotted on a log scale; $\log_{10}[\text{ISI(s)}]$. At DIV15, the first peak clusters around shorter ISIs and likely reflect high-frequency spikes within bursts. A second peak appears at 1 sec and likely reflects intervals between bursts. DMSO-treated networks exhibited increased short ISI frequency counts, while 1µM TO- and 5µM TO-treated networks exhibited decreased short ISI counts.
LXR Activation Suppresses Overall Spiking Activity and Bursting Activity

Coupled with the ISI data, the activity patterns observed in our raster plots led us to hypothesize that five days of TO901317 treatment would result in decreased total spike counts and decreased bursting activity in the MEA. One-way ANOVA detected a significant effect of LXR activation on total spike counts \( (F_{(2,7)} = 5.40, P < 0.05) \), with Dunnett’s multiple comparison test revealing a decrease in 5µM TO-treated spike counts relative to DMSO \( (P < 0.05) \) (Figure 34A). Analysis of total burst counts revealed a significant effect of TO901317 at DIV20 \( (F_{(2,7)} = 6.42, P < 0.05) \), with 5µM TO-treated networks exhibiting decreased bursts compared to DMSO-treated networks \( (5\mu\text{m TO}, P < 0.05) \) (Figure 34B). Treatment with TO901317 may have altered features of bursts in our hippocampal networks in addition to decreasing overall burst counts. Therefore, we analyzed the average duration of the bursts, the average number of spikes detected in bursts, and the average ISI within bursts. We did not detect significant differences between group means for average burst duration \( (F_{(2,7)} = 2.03, P > 0.05) \) (Figure 34C), average number of spikes in bursts \( (F_{(2,7)} = 3.42, P > 0.05) \) (Figure 34D), or average ISI within bursts \( (F_{(2,7)} = 0.53, P > 0.05) \) (data not shown).

Group means for all measures are displayed in Table 4 with \( P \) values. Importantly, no significant differences in any of our measures were detected between “grouped” MEAs at baseline (DIV15), suggesting that significant group differences in total spike counts and burst counts measured at DIV20 were due to TO901317 treatment, and not inherent differences in MEA network activity.
Figure 34. LXR activation suppresses total spikes and burst counts but does not alter burst duration or number of spikes in bursts. Hippocampal networks exhibit an important temporal pattern of spiking activity called bursts. Bursting activity can be detected in the MEA and quantified by defining two specific parameters: a minimum number of spikes per burst and a maximum cutoff for ISIs. Based on our ISI histograms, we defined bursts as containing no less than 4 spikes per burst with a maximum ISI of 100 ms (A) 5µM TO-treated networks exhibit decreased spike counts in the MEA. (B) 5µM TO treatment decreases total burst counts. (C-D) The average duration of bursts and spikes in bursts were not altered by drug treatment. All data are graphed as the percentage change from baseline measures. One-way ANOVA performed on DIV20 mean averages, with Dunnett’s post hoc test. 5µM TO vs DMSO, * P < 0.05.
<table>
<thead>
<tr>
<th>Dependent Measure</th>
<th>RX Group</th>
<th>DIV15</th>
<th>DIV20</th>
<th>One-way ANOVA P value (DIV20)</th>
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<tbody>
<tr>
<td>Total Spike Counts</td>
<td>DMSO</td>
<td>88031 ± 10456</td>
<td>106813 ± 31169</td>
<td>*0.0383</td>
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<tr>
<td></td>
<td>1µM TO</td>
<td>86894 ± 17884</td>
<td>41617 ± 11001</td>
<td></td>
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<tr>
<td></td>
<td>5µM TO</td>
<td>73687 ± 13345</td>
<td>32799 ± 4634</td>
<td></td>
</tr>
<tr>
<td>Total Burst Counts</td>
<td>DMSO</td>
<td>6617 ± 1216</td>
<td>8815 ± 2749</td>
<td>*0.0261</td>
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<tr>
<td></td>
<td>1µM TO</td>
<td>6043 ± 1280</td>
<td>2296 ± 614</td>
<td></td>
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<tr>
<td></td>
<td>5µM TO</td>
<td>4996 ± 1239</td>
<td>2069 ± 509</td>
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<tr>
<td>Avg Burst Duration</td>
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<td>61.91 ± 24.43 ms</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1µM TO</td>
<td>94.97 ± 28.93 ms</td>
<td>108.73 ± 39.57 ms</td>
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<td></td>
<td>5µM TO</td>
<td>86.87 ± 29.99 ms</td>
<td>42.52 ± 4.68 ms</td>
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<tr>
<td>Avg Spikes per Burst</td>
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<td>10.97 ± 0.59</td>
<td>8.43 ± 1.79</td>
<td>*0.0919</td>
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<td></td>
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<td>5µM TO</td>
<td>8.88 ± 1.18</td>
<td>8.66 ± 0.87</td>
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</tr>
</tbody>
</table>

Table 4. Group means for dependent measures in the MEA at baseline and DIV20. One-way ANOVA revealed significant differences in total spike counts and total burst counts at DIV20. There were no differences in features of bursts, including average burst duration (ms) or average number of spikes per burst. Dunnett’s multiple comparisons test for each ANOVA revealed decreased spike counts in 5µM TO-treated networks, and decreased burst counts in 1µM TO- and 5µM TO-treated networks compared to DMSO-treated networks.
C. Discussion

Using a reduced in vitro preparation, we found that LXR activation with TO901317 increased apoE levels in CM and altered the network activity of hippocampal neurons in the MEA by suppressing the frequency of short ISIs. To investigate this further, we performed burst analysis on our recordings in order to assess the impact of LXR activation on high-frequency spiking activity. The total number of recorded bursts in 5μM TO901317 treated cultures was decreased at DIV20 compared to vehicle controls, and was lower than the number of bursts detected at baseline (DIV15). No statistically significant differences in total burst counts were detected from hippocampal networks after DMSO treatment. Interestingly, LXR activation did not alter the average duration of the bursts, the average number of spikes per burst, or the average ISIs within bursts. These results demonstrate a role for TO901317 in inhibiting the natural expression of bursting activity in hippocampal networks, and help establish the MEA as an attractive in vitro model for future mechanistic studies.

The mechanisms underlying APOE4-associated AD risk are not fully understood. In Chapter IV, we provide evidence to suggest that cortical dendritic spine density and spatial cognition are negatively impacted in young APOE4 mice, independent of AD pathology and aging associated cognitive impairment (Rodriguez et al., 2013). Our results led us to hypothesize that apoE may be exerting isoform-dependent effects on neuronal circuits that subserve spatial learning and memory in mice. However, a basic understanding of apoE and its role in neuronal network activity patterns is lacking. Therefore, we first tested the effects of LXR activation on primary rat hippocampal network activity in the MEA. We chose to increase endogenous levels of apoE by treating hippocampal networks with TO901317, a non-steroidal synthetic LXR
agonist known to increase APOE and ABCA1 gene expression in the brains of AD mouse models (Koldamova et al., 2005; Riddell et al., 2007). ApoE levels increase in CM of control non-treated and DMSO-treated cultures by DIV20, likely due to proliferation of glial cells and increased cellular network maturity. However, five days of TO treatment increased CM apoE levels beyond vehicle treated controls, with 5µM TO inducing the greatest increase.

When we measured network activity at DIV20, we observed a robust impact of drug treatment on the number of network bursts in the MEA. Specifically, TO treatment suppressed the number of recorded bursts in the MEA without effecting burst duration, spikes in bursts, or ISIs within bursts. Spontaneous bursting activity is a natural phenomenon that is observed in neuronal cultures in vitro (Clifford et al., 2011; Van Pelt et al., 2004; Wagenaar et al., 2006) and in mammalian brain development (Chiu and Weliky, 2001; Leinekugel et al., 2002). While neuronal bursting activity may serve a number of functions in vivo (Buzsaki et al., 2002; Cooper, 2002; Lisman, 1997), Wagenaar et al (2006) suggest that bursts are important for information processing required for maturation of networks in vitro. Information encoded within the intervals between bursts may be lost across the network upon a sudden decrease in burst counts. We hypothesize that LXR driven burst suppression may inhibit the capacity of hippocampal networks to engage in neural operations that facilitate modulations in excitability, such as synaptic potentiation. Bursting dynamics in TO-treated hippocampal networks may be negatively impacted after chemical LTP (cLTP). Notably, cLTP increases overall network activity while increasing network regularity, and robustly impacts bursting dynamics in the MEA (Niedringhaus et al., 2013).
LXRs are an attractive target for therapeutics aimed at ameliorating AD pathology. In addition to increasing expression of products involved in cholesterol homeostasis, LXR activation suppresses AD-related inflammation by inactivating pro-inflammatory gene expression (Mandrekar-Colucci and Landreth, 2011; Qin et al., 2006; Zelcer et al., 2007). Considerable attention has been given to the literature describing its role in soluble Aβ clearance and memory retention in AD transgenic mice (Cramer et al., 2012; Jiang et al., 2008; Tesseur et al., 2013). However, very little is known about the effect of LXR activation on neuronal network activity patterns. Bexarotene was recently shown to decrease inter-ictal spike rates in two mouse models of hyper-excitability: Kv1.1 null mice and human APP expressing J20 mice (Bomben et al., 2014). Application of 10µM bexarotene was also shown to decrease the spike rates of transverse WT hippocampal slices bathed in extracellular potassium, which induces bursting activity (Bomben et al., 2014). Our data demonstrating that TO901317 decreases burst counts in hippocampal networks agrees with the general findings in Bomben et al (2014), and suggests that LXR/RXR activation may impact neural information processing in addition to previously identified cellular roles in cholesterol efflux.

Understanding how apoE affects the development and function of networks of neurons is critical to the development of preventative therapeutics that compensate for apoE4-related brain changes. The preliminary results from our MEA experiments suggest that LXR activation robustly affects the occurrence of spontaneous bursts detected in hippocampal networks without altering the number of spikes within bursts or average burst durations. The TO-induced suppression of bursting activity in vitro likely reflects changes to hippocampal network excitability, which may in turn influence dynamical activity after synaptic potentiation.
CHAPTER VI
OVERALL DISCUSSION

A. Summary of Results

APOE is a polymorphic gene in humans that significantly alters the risk of developing LOAD. The precise mechanisms underlying this increased risk are unclear; however, APOE-associated AD risk may be related to its synergistic effects on Aβ pathology and neuroinflammation. In Chapter III, I present evidence supporting the hypothesis that human APOE4 differentially impacts Aβ driven neuroinflammatory processes in the brains of 5xFAD mice (Figure 35). Morphological and biochemical markers of microglial reactivity were increased in the cortex of adult E4FAD mice relative to E3FADs, and E4FAD mice exhibited alterations in morphological profiles of Aβ plaques, supporting a previously published report in these mice (Youmans et al., 2012b). These data suggest that APOE4 may play a role in synergizing the individual impact of the apoE4 isoform on Aβ pathology and neuroinflammation, which may exacerbate AD pathogenesis.

In addition to these effects, APOE genotype may influence LOAD risk by influencing normal brain processes early in life and independent of AD-related pathology (Figure 35). In Chapter IV, I provide evidence suggesting that APOE4 negatively impacts spatial cognition and neuronal dendritic morphology in mice early in the lifespan. Young APOE4 mice exhibit spatial learning and memory deficits in the Barnes maze, and have reduced dendritic spine density in compartment-specific regions of MEC pyramidal neurons.
Figure 35. Thesis research objective and experimental aims. Exploring facets of APOE-ε4 driven AD susceptibility is the central theme of this dissertation, with an emphasis on the dysregulation of normal CNS functions that augment disease risk. The effects of APOE4 on normal brain functions were addressed in two contexts: in AD-related pathological conditions and in normal conditions. EFAD mice and APOE knock-in mice were used to address our experimental aims, which are organized into research tiers. In the AD-related pathological condition, adult E4FAD mice were found to have increased microglial reactivity at Aβ plaques (local) in the cortex compared to E3FAD mice, with increased IL-1β levels (global), suggesting a role for APOE genotype in Aβ-driven neuroinflammation. In normal conditions, APOE4 mice were found to have spatial learning and memory deficits compared to APOE3 mice, which was associated with deficits in dendritic spine density of MEC neurons.
APOE4-specific deficits in dendritic complexity may represent a structural component of a larger and more complex issue; that is, impaired signaling in neuronal circuits underlying cognitive behavior. Little is known about the direct impact of apoE isoforms on activity-induced synaptic plasticity, resulting in an information gap at the network level (Figure 36). To address this, it was first necessary to investigate the role that apoE expression plays in network signaling within a reduced preparation. In Chapter V, I present evidence that suggests LXR activation – which increases apoE expression - influences network activity and bursting activity of cultured hippocampal cells recorded in a multielectrode array system. While LXRs are an attractive therapeutic target in AD for their role in modulating cholesterol homeostasis, Aβ clearance, and inflammation, these data highlight the potential role that apoE modulation may play in neuronal network dynamics independent of AD pathology.
Figure 36. Network analysis in the MEA to bridge the information gap. An *in vitro* MEA system was used to assess the effects of LXR activation – which increases apoE expression - on network signaling. The studies described in Chapter V establish a foundation for mechanistic studies into the role apoE and its isoforms play in neuronal population activity, which will elucidate how APOE4-associated changes in dendritic spine density affect cognitive function in APOE mice.
B. A Role for APOE4 in Neuroinflammation

A toxic gain-of-function or a harmful loss-of-function?

The phenotypic diversity that describes morphological changes in microglia are known to have functional implications in the CNS (Hanisch and Kettenmann, 2007). The studies described in Chapter III suggest that APOE4 genotype differentially affects functional aspects of microglial activation at cortical Aβ plaques. However, we cannot conclude from our immunohistochemical data that activated microglia in E4FAD mice are harmful to surrounding cells or if their activity is ineffective in combating Aβ pathology. In order to determine whether increased microglial activation in E4FAD mice constitutes a toxic gain-of-function or a harmful loss-of-function, complimentary studies that assay a wide spectrum of pro-inflammatory and anti-inflammatory molecules in the EFAD brain need to be conducted. We detected a nearly two-fold increase in cortical IL-1β levels in E4FAD mice (Figure 9). Thus, we suspect that adult E4FAD mice would exhibit elevated measures of other pro-inflammatory cytokines in the cortex, which may include IL-6, TNFα, and interferon γ. Alternatively, E4FAD mice might exhibit decreased levels of anti-inflammatory molecules in the cortex, such as IL1-receptor antagonist, transforming growth factor β, and fractalkine. Multiplex protein assays that probe for a number of pro-inflammatory and anti-inflammatory molecules in the EFAD cortex would help elucidate the inflammatory milieu in this region, and shed additional light on the activation states of Aβ-associated microglia.

To investigate specific microglial responses to individual Aβ plaques in the cortex, it may be possible to combine IHC detection of plaques with chromogenic in situ hybridization (CISH) in sagittal EFAD brain sections. This approach has been successfully used to detect amplification
of the oncogene human epidermal growth factor receptor 2 (HER2/neu) in biopsied tissue, which serves as a biomarker for breast cancer (Reisenbichler et al., 2012; Tanner et al., 2000). Use of this strategy would allow us to visualize microglia surrounding individual plaques by using an HRP-conjugated MOAB2 antibody and Iba1, while conceivably identifying upregulated inflammatory mRNA expression in microglial cells residing within Aβ plaque domains. If multiplex assays determine an APOE-dependent difference in inflammatory profiles in the cortex, then subsequent experiments using combined IHC-CISH would shed light on the specific pro-inflammatory or anti-inflammatory phenotypes of microglia at Aβ plaques.

Finally, it would be useful to examine the co-localization of apoE isoforms with MOAB2-positive plaques in our adult EFAD mice. ApoE4 is hypothesized to exhibit poor anti-inflammatory effects relative to apoE3 and apoE2 by forming less stable apoE/Aβ complexes in vivo (Tai et al., 2013). Unstable, or reduced levels, of apoE/Aβ complexes in E4FAD mice suggest that less soluble Aβ is being cleared by apoE4, which may drive increases in oAβ and neuroinflammation in these animals (Tai et al., 2014). Namba et al (1991) were the first to show that apoE co-localizes with Aβ plaques. Our preliminary staining experiments show that an antibody for human apoE associates with MOAB2-positive plaques in the subiculum and cortex, demonstrating feasibility of co-localization analyses in EFAD brain sections (Figure 37). We would expect that E4FAD mice would exhibit decreased levels of apoE4 co-localized within Aβ plaques in the cortex compared to E2FAD and E3FAD mice.
Figure 37. ApoE co-localizes to Aβ plaques in the subiculum and cortex. (A) Fluorescence immunostaining in the subiculum of 6-month old EFAD mice revealed a high degree of co-localized apoE at Aβ plaques, which we examined using the Coloc 2 plugin in Fiji through confocal image stacks. Individual channels shown at top, merged image at bottom. Scale bars, 20µM. (B) ApoE co-localized to smaller Aβ plaques in the deep layers of cortex near the frontal pole. ApoE also stains glial-like cells in cortical regions that do not stain positive for MOAB2. Scale bars, 50µM.
A Dynamic Role for APOE4 in Neuroinflammation

Constant adjustment in microglial activation states is likely to occur as Aβ is created and deposited in the AD brain. We speculate that APOE4 plays a dynamic role in Aβ-induced neuroinflammation, and that this response cannot be easily categorized as a definitive “toxic gain-of-function” or “loss-of protective function”. APOE4 microglia may be primed to respond to Aβ, but be unable to effectively phagocytose and clear the peptides due to apoE4/Aβ complex instability. Without an effective means of clearance, Aβ would be expected to aggregate and result in hyper-activated microglia that release a host of pro-inflammatory factors.
C. Mediating Factors in APOE4-Associated Behavioral Deficits

Can differences in receptor signaling explain the behavioral deficits observed in APOE4 mice?

ApoE4 may differentially stimulate signaling cascades in microglia that result in an exaggerated pro-inflammatory response to Aβ. In the absence of Aβ pathology or aging-related CNS insults, we ask whether apoE4 differentially impacts neuronal function. As noted in Chapter I, apoE-associated lipoproteins bind members of the cell surface LDL receptor family in order to release cholesterol and triglycerides within cells. However, apoE binding also appears to initiate several independent signaling pathways in neurons that may influence more broad neuronal functions, including network signaling. This dual role for apoE has been referred to as “double duty” in the literature (Chen et al., 2010). In vitro experiments reveal that an apoE mimetic peptide, which consists of a tandem repeat of the receptor-binding domain of apoE, binds to ApoE receptors on primary cortical neurons and leads to tyrosine phosphorylation of Dab1 and the extracellular signal-regulated kinase ½ (ERK1/2) (Hoe et al., 2006). Phosphorylation of Dab1 may itself influence the phosphorylation of ApoER2 by the Src-family kinase Fyn, which may influence spine density and LTP (Teal C. Burrell, personal communication, March 2014). ApoE-induced activation of the ERK signaling pathway has been shown to increase levels of the transcription factor cyclic AMP response element-binding protein (CREB) (Ohkubo et al., 2001), an important mediator of synaptic plasticity.

Interestingly, cortical cultures treated with Reelin exhibit nuclear phosphorylation of CREB when incubated with HEK293-secreted ApoE2 or ApoE3 lipoproteins, but not ApoE4 (Chen et al., 2010). This isoform-dependent difference suggests that interaction with Reelin is an important factor to consider when evaluating the signaling properties of apoE. Additionally, the
region-specific cell population being evaluated needs be taken into account, as apoE-isoforms may have different signaling effects in different CNS regions. For example, CA1 homogenates prepared from human APOE4 mice exhibit increased levels of phosphorylated ERK compared to APOE3 mice (Korwek et al., 2009). Importantly, hippocampal slices from APOE4 mice exhibit increased CA1-LTP, in agreement with reports of LTP-induced CREB phosphorylation via the ERK signaling pathway (Davis et al., 2000).

When considered together, these studies offer a possible explanation as to why behavioral performance may vary in our APOE mice. ApoE4 signaling in the adult cortex may interfere with the effects of Reelin on NMDA receptor-mediated synaptic events. This could negatively impact ERK signaling and CREB phosphorylation, leading to deficits in cognitive behavior in the Barnes maze. Concurrently, apoE4 signaling may interact with Reelin by facilitating the phosphorylation of ERK in hippocampal circuits or other regions that mediate perseverative errors. In this sense, spatial learning and memory in APOE4 mice would be negatively impacted by the additive effect of region-specific signaling cascades: attenuation of the spatial memory trace in cortex and strengthening of competing, non-spatial perseverative errors in hippocampus. However, additional studies in APOE mice that explore learning-induced ERK signaling in cortex and hippocampus are warranted.

Can impaired receptor trafficking explain the behavioral deficits observed in APOE4 mice?

ApoER2 couples NMDA receptors through PSD-95 via an alternatively spliced exon-19 domain (Beffert et al., 2005), and calcium imaging experiments have shown that LRP1 activation
mediates calcium signaling through NMDA receptors (Bacskai et al., 2000). These studies support an interaction between apoE receptors and NMDA receptors at the synapse.

ApoE4 may negatively affect this receptor interaction via impaired receptor trafficking. Chen et al (2010) report that cortical neurons incubated with ApoE4 resulted in decreased surface expression of ApoER2 (Chen et al., 2010). This in turn blocked Reelin-dependent NMDA receptor phosphorylation, decreased surface expression of AMPA and NMDA receptor subunits, and prevented Reelin-induced CA1-LTP in hippocampal slices. Prolonged sequestration of ApoE receptors in endosomes may thus affect the surface expression and function of neuronal glutamate receptors differently in APOE4 mice, which could have a profound effect on cognitive behavior. However, there exist technical limitations to testing this hypothesis in vivo. An alternative in vitro approach would be to compare recordings from apoE-treated primary cortical neurons in the MEA after washing surface-bound apoE. If bound apoE4 reduces surface expression of ApoER2 and NMDA receptors by prolonging sequestration, then we would expect a delayed recovery of electrical activity from apoE4 treated cultures relative to the other isoforms. We also speculate that impaired receptor recycling in apoE4 treated cultures would drastically affect functional recovery of the network after a stimulation protocol.

*Can differences in regional dendritic spine sub-types explain the behavioral deficits observed in APOE4 mice?*

APOE4 is associated with reduced numbers of stable, mature spines on basal shaft dendrites of CA1 pyramidal neurons (Jain et al., 2013). In the EC, APOE4 mice exhibit decreased numbers of thin spines along apical dendrites. These results suggest that deficits in
morphologically distinct spine-subtypes may be an important factor when resolving APOE4-associated behavioral impairments, in addition to general dendritic length and spine density measures (Dumanis et al., 2009; Rodriguez et al., 2013). Stubby, mushroom shaped spines have more AMPA receptors anchored into relatively larger post-synaptic densities than thin spines, allowing for stronger synaptic function that is likely to remain stable over time (Bourne and Harris, 2007). In contrast, thinner spines have smaller post-synaptic densities that contain less AMPA receptors, and have a higher turnover rate. Thin spines are also sensitive to modulations in synaptic activity (e.g. LTP and LTD), which respond by expanding or retracting their spine heads to accommodate newly enhanced or weakened input (Bourne and Harris, 2007).

Lower numbers of transient, thin spines on APOE4 entorhinal neurons may be partly responsible for behavioral deficits observed in our mice. Though we do not know the laminar organization of analyzed entorhinal neurons in this report (Jain et al., 2013), EC apical dendrites receive strong collateral innervation from neighboring neurons (Canto et al., 2008; Quilichini et al., 2010; Witter and Moser, 2006). A loss of highly adaptable, thin spines on these cells may result in disturbed information processing required for the acquisition of a spatial task, like in the Barnes maze. The lower numbers of stubby, mushroom shaped spines on APOE4 CA1 neurons may also contribute to APOE4-associated behavioral deficits. Both basal and proximal apical dendrites of CA1 neurons receive input from adjacent CA3 cells in the hippocampus. We hypothesize that fewer mature, stable spines on CA1 dendrites may interfere with the consolidation of spatial information acquired during a task. This would place the APOE4 mice at a considerable cognitive disadvantage relative to APOE3 mice, as spatial acquisition may have already been compromised upstream in the circuit (EC). Taken together, the robust effect of
APOE4 on neuronal dendritic length, spine density, and spine sub-types provides a hint at a larger, more complex issue. Structural deficits in neurons may impede the activity of neuronal circuits that govern cognitive processes, leaving human APOE-ε4 carriers at an increased susceptibility for AD.

**APOE genotype and spatially modulated cells in the brain: A new perspective?**

Structural changes to dendritic spines and poor receptor recycling may have a combined effect on spatially modulated neurons in the MEC and hippocampus of APOE4 mice. This intriguing possibility would suggest that complex spiking neurons in circuits that subserve spatial navigation and cognition are sensitive to the effects of apoE isoforms, which may further explain the behavioral deficits observed in our APOE4 mice. We suspect that exposure to the Barnes maze activates a network of grid cells in the MEC and place cells in the hippocampus of our mice, with MEC grid cells supporting context-independent path integration and CA1 place cells supporting context-dependent episodic memory (Barry and Bush, 2012; Bush et al., 2014; Fyhn et al., 2004; Hafting et al., 2005). Importantly, disruption in place cell firing is associated with poor behavioral performance in the water maze (Liu et al., 2003). We hypothesize that apoE4 reduces the availability of surface ApoER2 and glutamate receptors at dendritic spines on CA1 place cells, resulting in inadequate information processing and subtle deficits in spatial behavior.
D. Effects of ApoE Isoforms on Network Activity in the MEA

My overarching hypothesis is that APOE genotype affects normal brain function before AD pathogenesis, specifically by affecting the development and network activity of organized neuronal populations in the brain. In the absence of hallmark AD pathology, young (4-weeks to 8-month old) and older (9- to 24-month old) APOE4 mice exhibit reduced dendritic spine density in pyramidal neurons of the somatosensory cortex, MEC, and the DG and CA1 regions of the hippocampus (Dumanis et al., 2009; Jain et al., 2013; Ji et al., 2003; Rodriguez et al., 2013). APOE4 mice also exhibit alterations in the glutamate-glutamine cycle within pre-synaptic terminals (Dumanis et al., 2013), suggesting that poor CNS function in these mice may result from impaired excitatory neurotransmission in activated neuronal circuits that subserve cognitive behaviors. Unfortunately, very few studies have tested the direct influence of natively expressed apoE isoforms on activity-induced synaptic plasticity (Chen et al., 2010; Trommer et al., 2004; Wang et al., 2005), resulting in an information gap at the neuronal network level. Moreover, an optimal experimental model to test the effects of native apoE on network organization and neuronal activity has not been established.

Assessing the impact of apoE isoforms on network activity: A new approach using the MEA

In Chapter V, we cultured primary rat hippocampal cells in MEAs and tested the effects of LXR activation on neuronal network activity. These preliminary experiments were conducted to elucidate the basic neurobiological functions of apoE on hippocampal networks before and after synaptic potentiation. Further experiments that directly test the influence of apoE on
bursting activity will be carried out by recording from TO901317-treated hippocampal networks derived from APOE\textsuperscript{−/−} rats (Sage Labs, Boyertown, PA).

In principle, similar experiments that test the direct effects of apoE isoforms on neuronal network activity can be conducted in the MEA. By culturing primary hippocampal cells from human APOE mouse lines, we can directly test the effects of individual apoE isoforms on neuronal network organization and spiking activity patterns prior to and following pharmacological stimulation. We have developed a modified protocol for culturing primary hippocampal and cortical cells from each line of homozygous APOE mice in the MEA. These healthy, mixed-cell cultures develop dense cellular networks within the MEA over days in vitro and secrete detectable levels of apoE into the CM (Figure 38). This in vitro culture system provides a basis to begin experiments that test mechanistically how individual apoE isoforms influence neuronal network activity.
Figure 38. In vitro APOE4 mixed-cell cultures develop networks and secrete detectable levels of apoE4. (A) Above, Secreted apoE4 is detected in CM at DIV7 and DIV14 by Western blot (2\textsuperscript{nd} and 3\textsuperscript{rd} lanes). 1\textsuperscript{st} lane is a negative control (maintenance media). 4\textsuperscript{th} lane is a positive control (recombinant apoE4). Below, APOE4 cortical cells in the MEA active zone at DIV10. Scale bar, 200µM. (B-C) Healthy neuronal (MAP2, red) and astrocyte (GFAP, green) processes are evident in APOE4 cortical and hippocampal cultures. Cell nuclei are stained with DAPI (blue). Scale bars, 30µM.
Previous *in vitro* work has demonstrated that apoE4 delays dendritic spine formation by DIV14, and that apoE4 is associated with an accelerated loss of mature spines at DIV21 (Nwabuisi-Heath et al., 2014). Importantly, these experiments utilized a primary cell non-contact co-culture system in which DIV5 neurons harvested from C57BL/6J mice were grown in the presence of natively secreted human apoE isoforms (Nwabuisi-Heath et al., 2014). We would expect similar results on dendritic spine formation and maturation within our primary mixed-cell APOE4 networks, as natively expressed apoE4 would likely be undisturbed in this system. However, a key difference in our experimental approach would be that astrocytes are in direct contact with neurons, allowing bidirectional communication between astrocytes and neurons that may influence signaling in the MEA. Indeed, information exchange occurs between astrocytes and neurons at “tripartite” synapses, which refer to the close apposition of synaptic structures by astrocytic processes (Perea et al., 2009). Molecular diffusion is relatively fast and free in the distal ends of astrocytic processes, which differ from the more restricted diffusion dynamics seen in the endfeet that contact blood vessels (Nuriya and Yasui, 2013). This suggests that signaling molecules at the synapse can influence neighboring cells within seconds, and may serve as a critical regulatory mechanism in homeostatic control over a population of nearby neurons. We hypothesize that the fast bidirectional communication between astrocytes and neurons at tripartite synapses would be lost in a mixed-cell, non-contact co-culture system, and would therefore be unsuitable for studies of spontaneous network activity. These features would likely be preserved in our mixed-cell cultures, where astrocytes contact neurons and integrate within neuronal networks in the MEA.
CHAPTER VII
CONCLUSIONS

The total number of AD cases in the US is projected to reach 13.8 million by the year 2050 and result in a total annual cost of $1.2 trillion (in 2014 dollars) (Hebert et al., 2013). Without an effective way to manage the symptoms, slow the progression, or prevent AD onset, Alzheimer’s disease will remain one of the top 10 killers in this country and continue to devastate the lives of loved ones left behind.

The experimental studies described here offer new perspectives on the function of APOE – the strongest genetic risk factor for AD – in both AD-related pathological conditions and in normal brain functions. We utilized a newly described mouse model of AD risk and Aβ deposition (EFAD) to reveal a role for APOE genotype in Aβ-associated microglial activation in the cortex (Rodriguez et al., 2014). In our AD risk model (APOE targeted replacement), we found that neuronal dendritic morphology and spatial cognition are differentially modulated by APOE genotype at a relatively young age, independent of AD pathology (Rodriguez et al., 2013). We conclude that \textit{APOE}-\textit{ε4}, the allele associated with increased AD risk, exacerbates AD pathology by mounting an exaggerated and ineffective microglial response at Aβ plaques in the brain. We speculate that a chronic, pro-inflammatory milieu leads to downstream effects on nearby neurons, and may contribute to region-specific AD neurodegeneration in human \textit{APOE}-\textit{ε4} carriers. Additionally, we conclude that APOE4 affects structural components of cortical projection neurons that innervate the hippocampus, and that this effect may be partly responsible for subtle deficits in spatial cognition detected in human \textit{APOE}-\textit{ε4} carriers prior to AD onset.
APOE4 may thus confer increased susceptibility for AD by negatively impacting normal neuronal functions, which puts aging APOE-ε4 carriers at a considerable disadvantage to APOE-ε2 and APOE-ε3 individuals.

In conclusion, our results reveal a need for APOE-targeted therapeutics in AD treatment and prevention that are sensitive to APOE allele status. Increased investment in translational efforts that mitigate the impact of APOE4 on AD-related Aβ and neuroinflammation are sorely needed. In addition, concurrent investment in basic research that contributes to a rich, mechanistic understanding of APOE in a variety of CNS functions is required to move treatments from the bench to the bedside.
CHAPTER VIII
REFERENCES


retention deficits on a wide range of spatial memory tasks. Behavioural brain research 193, 174-182.


