CONSEQUENCES OF APOLIPOPROTEIN E ISOFORM VARIATION: EFFECTS ON HIPPOCAMPUS SYNAPTIC PLASTICITY, LEARNING AND MEMORY IN THE ADULT MOUSE

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LIST OF ABBREVIATIONS

AD ....Alzheimer's Disease

Aβ..........beta amyloid

AICD ....APP intracellular domain

α2M alpha-2-macroglobulin

AMPA..........α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

apoE..........apolipoprotein E

apoE TR ....apolipoprotein E targeted replacement

apoER2..........apolipoprotein E receptor 2

APP..........amyloid precursor protein

APV..........2-amino-5-phosphonovaleric acid

BACE..........beta site APP cleaving enzyme

CamKII..........calcium-calmodulin dependent protein kinase II

CDK5 ....cyclin-dependent kinase 5

CNS ....central nervous system

CREB ....cAMP response element binding

CS ....conditioned stimulus

CSF ....cerebral spinal fluid

Dab-1 ....disabled-1

EGF ....epidermal growth factor

ERK ....extracellular-regulated kinase

FAD .... familial Alzheimer's disease

fEPSP .... field excitatory postsynaptic potential

GSK-3β .... glycogen synthase kinase 3 beta

HDL .... high density lipoprotein

HRM .... heterozygous reeler mouse

ICD .... intracellular domain

JIP .... JNK interacting protein

JNK .... c-jun N-terminal kinase

LDL .... low density lipoprotein

LDLR .... low density lipoprotein receptor

LRP .... low density lipoprotein-related protein

LTP .... long term potentiation

MAP2 .... microtubule associated protein 2

MAPK .... mitogen-activated protein kinase

MEGF7 .... multipled EGF containing protein 7

MEK .... mitogen-activated protein kinase kinase

NFT .... neurofibrillary tangle

NMDA .... N-methyl-D-aspartic acid

PI3K phosphatidylinostiol-3-kinase

PKB .... protein kinase B

PPI .... prepulse inhibition

PS1 .... presenilin 1

PS2 ....presenilin 2

PTB ....phosphotyrosine-binding

RAP ....receptor associated protein

rhapoE...recombinant human apolipoprotein E

sAPP ....soluble amyloid precursor protein

Sepp-1 ....selenoprotein P-1

SFK...src-family tyrosine kinase

SORLA ....sorting protein-related receptor

SYN ....synaptophysin

TBS...theta burst stimulation

TGFα..transforming growth factor-alpha

tPA ....tissue plasminogen activator

US ....unconditioned stimulus

VLDL...very low density lipoprotein

VLDLR..very low density lipoprotein receptor

CHAPTER I

INTRODUCTION

Our abilities to learn, remember, adapt and respond to our environment are highly regulated processes essential to survival. Small alterations to these vital systems can have a huge impact on cognitive function and quality of life. When such alterations occur, they may manifest as neurological disease, giving us a glimpse into the complicated workings of learning and memory. Studying neurological diseases, such as Alzheimer's disease, both progresses therapeutic research and identifies those integral components that make our everyday functioning possible.

Alzheimer's disease (AD) is a devastating form of dementia characterized by specific pathological hallmarks, namely beta amyloid (Aβ) plaques and neurofibrillary tangles (NFTs). The extracellular deposits of Aβ, a product of the cleavage of amyloid precursor protein (APP), distinguish AD from other forms of dementia. These Aβ plaques are accompanied by NFTs, which are intracellular accumulations of the microtubule stabilizing protein tau, and a specific pattern of neuronal cell death. This pattern of cell death results in the characteristic and progressive deficits in cognitive abilities and memory that interfere with daily function.

One of the first brain structures affected in AD is the hippocampal formation. Composed of the dentate gyrus, hippocampus proper, and subiculum, this structure is a key component of memory processing (Eichenbaum, et al., 1996). Together with

surrounding cortical areas in the temporal lobe such as the parahippocampal cortex, entorhinal cortex, and perihinal cortex, the hippocampal formation is essential for the formation of spatial and episodic memory (Eichenbaum, 1999; LeDoux, et al., 1990; Nagahara, et al., 1995; Phillips & LeDoux, 1992). Early AD is characterized by a specific pattern of memory loss and cognitive disfunction that results from loss of function in the entorhinal cortex and CA1 subfield (Braak & Braak, 1998; Ohm, et al., 2003). As the disease advances, neurodegeneration spreads progressively to include the entire hippocampal formation and increasing amounts of the surrounding cortex (Braak & Braak, 1998). While the pathology of AD is well established, the etiology of the disease remains elusive.

It is the formation of Aβ that is thought to direct the pathology of AD, forming the basis for the "Aβ cascade hypothesis" of disease progression (Selkoe, 1991). This hypothesis suggests that aberrant processing of APP results in overproduction of Aβ. This event then drives the formation of amyloid plaques, induces inflammation due to activation of astrocytes and microglia, and initiates development of neurofibrillary tangles due to alterations in cell signaling (Hardy & Allsop, 1991; Selkoe, 1991, 2002a; Tanzi, 2005). These processes ultimately result in the synaptic and neuronal injury that cause cell death and dementia.

The Aβ hypothesis of AD developed in part due to studies of familial AD. Early-onset familial AD is a hereditary form of AD that occurs prior to age 60. This form of the disease stems from mutations in three known genes: APP, presenilin-1 (PS1), and presenilin-2 (PS2). Mutations in PS1 and PS2, which cleave APP to form Aβ, selectively

increase levels of Aβ42 over Aβ40 (Borchelt, et al., 1996; Citron, et al., 1997; Duff, et al., 1996; Scheuner, et al., 1996). Aβ42 is the form of Aβ that is more highly prone to oligomerization and plaque formation (Bitan, et al., 2003; Burdick, et al., 1992; Jarrett, et al., 1993). Inherited mutations in APP also increase Aβ production by altering processing of APP (Citron, et al., 1992; Goate, et al., 1991; Levy, et al., 1990; Suzuki, et al., 1994). Several of these human APP mutations have been translated into transgenic mice and display alterations in Aβ production or accumulation. For example, the 5X FAD mouse, a mouse expressing 5 familial AD (FAD) mutations including both human APP and PS1 mutations, has Aβ42 accumulation at 1.5 months followed by synapse degeneration, neuron loss, and spatial learning deficits (Oakley, et al., 2006). Mice expressing the Swedish double mutation (K595N/M596L) of human APP, also known as APP695Swe or Tg2576, also have numerous Aβ plaques accompanied by memory deficits by 9-10 months of age (Hsiao, et al., 1996; Ribe, et al., 2005; Westerman, et al., 2002). The PDAPP mouse, which expresses human APP mutation V717F under the human platelet derived growth factor b (PDGF-b) promoter, has significantly increased Aβ levels, hippocampal atrophy, and impairments in learning and memory (G. Chen, et al., 2000; Games, et al., 1995; Gerlai, et al., 2001; Rockenstein, et al., 1995).

While these transgenic models of AD help support the Aβ cascade hypothesis, this hypothesis is not without controversy. The clinical progression of AD does not show a simple correlative relationship with the amount or temporal progression of amyloid deposits in the brain, making them of limited use for predicting neuropathological stages (Braak & Braak, 1998). Likewise, Aβ can be found in the brains of the elderly without

associated dementia (Knopman, et al., 2003). Furthermore, the majority of AD cases are classified at late onset sporadic AD. While all the pathological hallmarks are present, this form of AD is not associated with any of the autosomal dominant mutations present in familial AD. Instead, the only known genetic risk factor for AD is allelic variation in apolipoprotein E (apoE). This variation will be discussed in detail in the following section, but it is worthwhile to note that while the ε4 allele of apoE increases cerebral Aβ burden in late-onset AD patients (Rebeck, et al., 1993; Schmechel, et al., 1993), possession of this allele is not a causative factor in AD development. This suggests that modulatory factors can alter neuronal function prior to the formation of Aβ plaques, NFTs, or the onset of clinical AD.

Recent research has identified apoE as a potential modulator of neuronal function. ApoE, and its receptors, associate with amyloid plaques (Arelin, et al., 2002; Motoi, et al., 2004). Further research identified a differential impact of the three human isoforms of apoE—apoE2, apoE3, and apoE4—on AD risk (Rebeck, et al., 1993; Schmechel, et al., 1993). These three human isoforms of apoE have the potential to alter a number of neuronal processes that impact AD development. This includes inflammation (Guo, et al., 2004; Koistinaho & Koistinaho, 2005; LaDu, et al., 2001), Aβ production (Fagan, et al., 2002; Irizarry, et al., 2004) and deposition (Bales, et al., 1999; DeMattos, 2004), or signal transduction (Riemenschneider, et al., 2002; Trommer, et al., 2005). The role of apoE in modulating signal transduction is supported by recent research identifying the necessity of the low density lipoprotein receptor (LDLR) family of apoE receptors in

maintaing proper synaptic plasticity, and the propensity for dysfunction in signaling by these receptors to lead to cognitive disorders.

The low density lipoprotein receptor family

The LDLR family is an evolutionarily conserved group of multifunctional cell surface proteins with analogs found in *Aplysia*, *C. elegans* and *Drosophila* (Marx, 1985; Schonbaum, et al., 1995; Yochem & Greenwald, 1993). The LDLR family consists of seven known mammalian members: low-density-lipoprotein receptor (LDLR), very-low-density-lipoprotein receptor (VLDLR), apolipoprotein E receptor 2 (apoER2), multiple epidermal growth factor (EGF) repeat-containing protein-7 (MEGF7), LDL-related protein (LRP), LDL-related protein-1B (LRP-1B) and megalin (figure 1). There are also other related receptors that share some, but not all, of the structural hallmarks of the LDLR family, including LRP5/LRP6 and sorting protein-related receptor (SORLA) (Beffert, Stolt, et al., 2004; Herz & Bock, 2002; Taira, et al., 2001).

The LDLR family is characterized by shared structural elements (figure 1). Each receptor contains a ligand binding domain that mediates the interaction between the receptor and apoB-100/apoE containing lipoproteins. Additionally, each receptor also contains an EGF precursor homology domain, a YWTD β-propellor motif, a single transmembrane domain, and at least one NPxY motif on the cytoplasmic tail. The tetra-amino acid NPxY motif is a putative interaction site for intracellular adaptor proteins and couples LDL receptors to specific signal transduction pathways (Nimpf & Schneider, 2000; Trommsdorff, et al., 1999; Willnow, 1999; Willnow, et al., 1999). These pathways

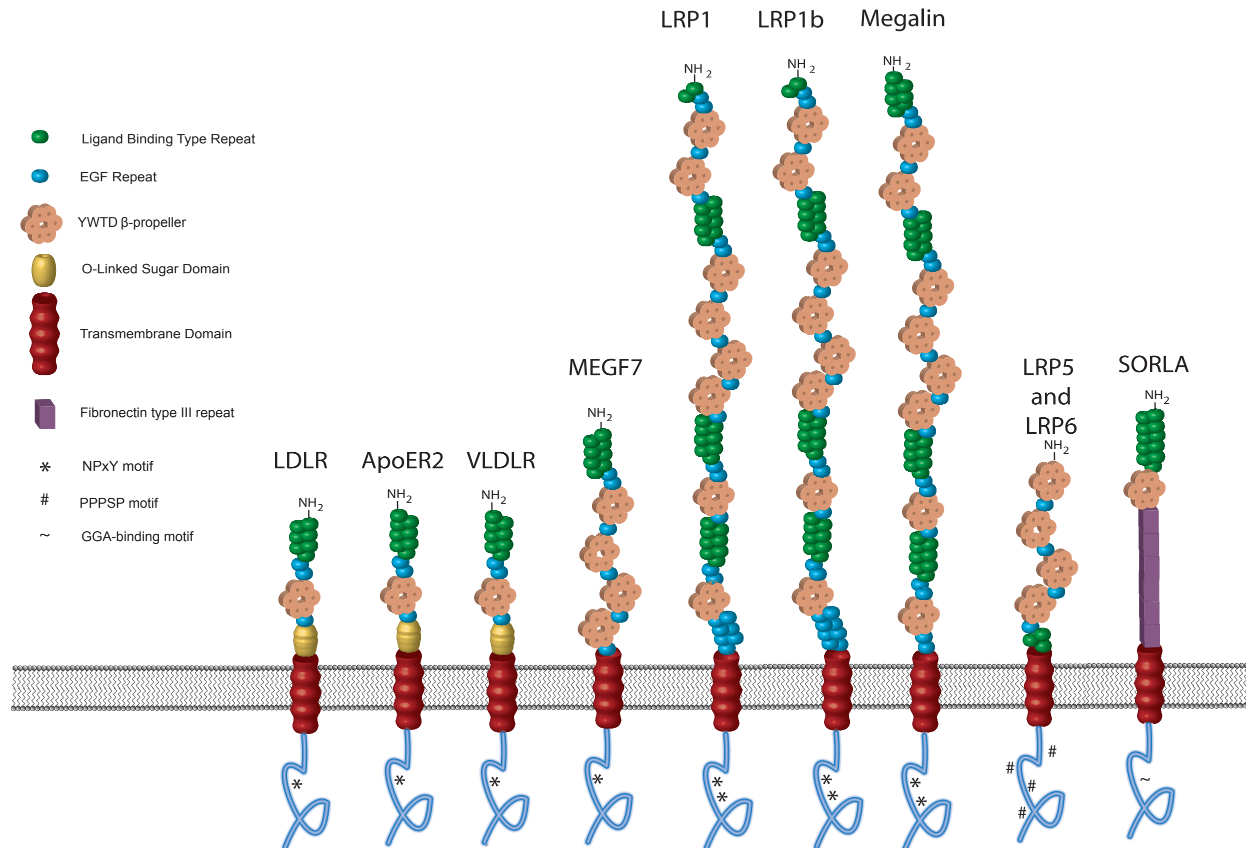


Figure 1: The low density lipoprotein receptor (LDLR) family
The LDLR family of apoE receptors share structural features including a single transmembrane domain, a short cytoplasmic tail, ligand-binding repeats (also called complement-type repeats), epidermal growth factor (EGF) repeats and YWTD-containing β-propeller domains.

can enlist MAP kinases (Senokuchi, et al., 2004), tyrosine kinases (Bock & Herz, 2003; Gouni-Berthold & Sachinidis, 2004), lipid kinases (Bock, et al., 2003) and ligand-gated ion channels (e.g. glutamate receptors (Beffert, et al., 2005; S. Qiu & Weeber, 2007; Sinagra, et al., 2005)). Through these signaling events, the LDLR family plays a role in a variety of cellular functions such as extracellular protein endocytosis, cross-membrane signal transduction, and modulation of synaptic function.

The function of the various LDLR family members are defined, in part, by the restriction of expression of individual receptor to particular tissues. The LDL receptor is highly expressed in the liver where it mediates endocytosis of lipoprotein particles (Goldstein, et al., 1985; Jeon & Blacklow, 2005). This receptor is also expressed in the brain where there is greater expression in glia than neurons (Herz & Bock, 2002). LRP1 is also found in most tissues, and is abundant in hepatocytes and neurons (May, et al., 2004). LRP1b is primarily expressed in the brain, but may also act as a tumor suppressor gene. This speculative function is suggested by inactivating mutations in LRP1b in lung cancer cell lines (Liu, et al., 2001). Megalin, structurally similar to LRP and LRP1b, is highly expressed in the epithelial cells that line the proximal tubes in the kidney (Willnow, 1999), with expression in the brain as well. Unlike LDLR, VLDLR is not expressed in the liver, but is instead restricted to the heart, skeletal muscle, endothelial cells of major blood vessels, and the brain (Herz & Bock, 2002). ApoER2, similarly, is not found in the liver, and expression of this receptor is almost exclusively limited to the testes and brain (Herz & Bock, 2002).

In addition to their structural similarities, members of the LDLR family are also characterized by shared ligands, namely receptor-associated protein (RAP) and apoE. RAP is a molecular chaperone that binds to LDL receptors in the endoplasmic reticulum, preventing the premature binding of ligands and potentially assisting in folding (Bu & Schwartz, 1998). While RAP is not endogenously found in the extracellular space, it is a useful experimental tool as it antagonizes ligand binding to all LDL receptors (Bu, 1998). The endogenous extracellular ligand apoE binds to all the major LDLR family members in the brain, LDLR, VLDLR, ApoER2, LRP1, and LRP1b. However, these receptors and the remaining LDLR family members are distinguished in part by differential ligand binding (table 1, (Bu, 2009; Herz & Bock, 2002; Herz & Strickland, 2001; May, et al., 2005)).

The LDL receptor is the primary receptor for cholesterol homeostasis due to the strong interactions with both apoB-100- and apoE-containing lipoproteins (Goldstein, et al., 1985; Jeon & Blacklow, 2005). Mutations leading to the loss of LDLR function results in familial hypercholesterolemia and atherosclerosis. LRP1 also undergoes endocytosis to transport ligands across the cell membrane. In addition to apoE, LRP1 binds many other ligands including alpha-2-macrogloblin (α2M), tissue plasminogen activator (tPA), APP, Aβ, and many others (Herz & Strickland, 2001). These interactions implicate LRP1 in a variety of cellular processes beyond cholesterol transport such as APP trafficking, Aβ clearance, and coagulation regulation (Herz & Strickland, 2001; Rebeck, et al., 2001; Shibata, et al., 2000; Ulery & Strickland, 2000). The ~600kDa

Table 1: Differential ligand binding and biological functions of LDLR family members

Receptor	Ligands	Primary biological functions
LDLR[1,2,3,5,8]	apoB, apoE	transport of cholesterol and lipoproteins, clearance of Aβ (via apoE/Aβ complex)
apoER2[1,3,8]	reelin, apoE, APP, F-spondin, selenoprotein P	neuronal migration and cortical lamination, synaptic transmission, APP trafficking, male fertility
VLDLR[1,3,8]	reelin, apoE, lipoprotein lipase, tPA	neuronal migration and cortical lamination, synaptic transmission
MEGF7[1]	apoE, agrin	limb patterning, formation of neuromuscular synapses
LRP1[1,3,4,7,8]	apoE, chylomicron remnants, α2M, tPA, APP, Aβ, protease/protease inhibitor complexes, lipoprotein lipase, PDGF, TGFβ, MMP-9	endocytosis and metabolism of various ligands, synaptic transmission, APP trafficking, clearance of Aβ, embryonic development, blood coagulation, angiogenesis
LRP1b[1,6]	likely overlaps with LRP1	potential tumor suppressor
Megalin[1,3,8,9]	apoB, apoE, apoJ, albumin, cubilin, retinol-binding protein, Vitamin D-binding protein, sonic hedgehog, BMP-4	Nutrient resorption in kidney, sonic hedgehog signaling, embryonic cholesterol homeostasis, calcium homeostasis
LRP5 and LRP6[1,3]	Wnts, Dkk1, Wise, SOST, R-spondin	Wnt co-receptor, embryonic development, regulation of bone formation, stem cell maintenance and survival
SORLA[1,3]	APP, apoE, head activator peptide	APP trafficking, neurogenesis, intracellular trafficking in the kidney

[1]Bu, et al., 2009
[2]Goldstein, et al., 1985
[3]Herz & Bock 2002
[4]Herz & Strickland, 2001
[5]Jeon & Blacklow, 2005
[6]Liu et al., 2001
[7]May, et al., 2004
[8]May, et al., 2005
[9]Willnow, et al., 1999

LRP1 is essential for early embryonic development, as deletion of the *Lrp1* gene in mice is embryonic lethal (Herz, et al., 1992). LRP1 is also implicated in synaptic transmission and motor function in the central nervous system (CNS), as forebrain-specific knockout of *Lrp1* results in a functional deficit without neurodegeneration (May, et al., 2004).

In addition to the endocytic properties of LDLR and LRP1, apoE receptors also couple with intracellular signal transduction cascades. Most notable is the interaction of reelin with apoER2 and VLDLR to trigger signaling crucial to neuronal migration (Trommsdorff, et al., 1999), dendritic spine development (Niu, et al., 2004), and synaptic plasticity (Beffert, et al., 2005; Beffert, Weeber, et al., 2004). Reelin signaling will be discussed in depth later in this chapter. The remaining members of the LDLR family share structural similarities and overlapping ligand binding properties, but have a wide range of functions in various tissues. This includes nutrient reabsorption in the kidneys (megalin) (Willnow, et al., 1999), potential tumor suppression (LRP1B) (Liu, et al., 2001), limb patterning (MEGF7) (Johnson, et al., 2005) and Wnt-β-catenin signaling (LRP5 and LRP6) (He, et al., 2004). While these receptors and their function are beyond the scope of this project, they have been extensively studied and are the subject of a number of reviews (Beffert, Stolt, et al., 2004; Herz, 2009; Herz & Bock, 2002; Herz & Strickland, 2001; May, et al., 2005; S. Qiu, Korwek, & Weeber, 2006; Willnow, et al., 1999).

The four major apoE receptors in the CNS—LDLR, apoER2, VLDLR, and LRP1—can undergo cleavage to produce soluble forms of the receptors. LRP1 is synthesized as a single ~600 kDa polypeptide that is cleaved by furin in the trans-Golgi network. The

515 kDa ligand-binding portion and the 85 kDa transmembrane portion remain non-covalently associated as the receptor is trafficked to the cell surface (Herz, et al., 1990). Subsequently, the extracellular domain is cleaved by metalloproteinases, producing a soluble form of the receptor with ligand binding properties (Grimsley, et al., 1998; K. A. Quinn, et al., 1997). Further cleavage by γ-secretase within the transmembrane domain releases the intracellular domain (ICD) (Kinoshita, et al., 2003; May, et al., 2002). ApoER2 and VLDLR are processed in a similar fashion. Phorbol ester application can cause cleavage by metalloproteinases to release soluble receptors (Hoe & Rebeck, 2005), and the remaining C-terminal fragments are also cleaved by γ-secretase (May, et al., 2003). Interestingly, the cleavage of apoER2 and VLDLR can be altered by apoE isoform, with apoE2 inducing the greatest cleavage, apoE3 slightly less, and apoE4 the least (Hoe & Rebeck, 2005). LDLR also undergoes metalloproteinase-dependent cleavage to produce a soluble form similar to apoER2, VLDLR, and LRP1 (Begg, et al., 2004).

The cleavage of lipoprotein receptors has the potential to alter neuronal function through modulation of cellular signaling. While the physiologic consequences of soluble apoE receptors is still under investigation, the function of released extracellular domains of other proteins hints at the possibility of similar cellular roles for soluble lipoprotein receptors. This includes the ability to function at long distances from the cell similar to transforming growth factor-alpha (TGF-α) (Arribas & Massague, 1995), initiation of intracellular signaling by the release of the cytoplasmic domain as seen with the Notch receptor (Mumm, et al., 2000), and inhibition of signaling by binding of ligands, a

mechanism observed with several interleukins (Arend, et al., 1994; Fernandez-Botran, et al., 1996; Mortier, et al., 2004). Inhibition of ligand binding has been shown with soluble apoER2, which blocks binding of the ligand reelin to apoER2 and VLDLR. The physiological importance of signaling cascades normally initiated by this binding emphasizes the necessity for tightly controlled modulation of lipoprotein receptor function.

Signaling by lipoprotein receptors

The LDLR family can induce alterations in cellular signaling due to coupling with intracellular effectors, such as MAP kinases, tyrosine kinases, or ion channels. The NPxY motif on the cytoplasmic tails of these receptors mediates endocytosis and signaling of these receptors via intracellular adapter proteins. There are an array of intracellular ligands for the LDLR family that help couple these receptors to signaling cascades (table 2, for review see (Gotthardt, et al., 2000; Herz & Bock, 2002; Herz & Strickland, 2001)). The physiological significance of these ligands continues to be elucidated. One interaction that has been characterized is that of Disabled-1 (Dab-1) with apoER2 and VLDLR to mediate reelin signaling.

ApoER2 and VLDLR exclusively mediate the binding of the extracellular matrix protein reelin. Reelin, a ~400 kDa glycoprotein, was identified as the protein deleted in the *reeler* mutant mouse line (D'Arcangelo, et al., 1995). This mouse displays a severely ataxic gait and failure of proper cortical lamination development. During neocortical

Table 2: Intracellular ligands for the LDL receptor family

Ligand	Function
Disabled-1 (Dab-1)[1,2,3,5]	Activation of Src family kinases, neuronal migration
Disabled-2 (Dab-2)[2,3]	Endocytosis, vesicular trafficking
JIP-1/JIP-2[1,2,3]	Regulation of MAPK and SAPK, including JNK
PSD-95[1,2,3]	Scaffolding in post synaptic density, coupling to NMDARs
Axin[2,3]	Wnt signaling
Fe65[2,3,4]	APP processing, actin remodeling
OMP25[2,3]	Mitochondrial transport
PIP4,5 kinase like protein[2,3]	Regulation of inositol signaling
CAPON[1,2,3]	Regulation of NO synthase
SEMCAP-1[1,2,3]	Possible axon guidance, vesicular transport
Shc[2,3]	Activation of Ras

[1]Gotthardt, et al. 2000
[2]Herz & Bock, 2002
[3]Herz & Strickland, 2001
[4]Hoe et al., 2006
[5]Howell et al., 1997

development, Cajal-Retizus cells in the pre-plate synthesize and express reelin. Reelin is necessary for the signaling that controls neuronal migration along the radial glia as deletion of reelin results in a failure of the pre-plate to split. Each new generation of cells also fails to migrate past the previously born cells to form the characteristic “inside-out” lamination of the cortex (Tissir & Goffinet, 2003). Severe neuronal migration defects and improper lamination are seen in the hippocampus, cerebellum, and cortex. Absence of both apoER2 and VLDLR or the intracellular adapter protein Dab-1 mimics the developmental phenotype seen with reelin deletion (D'Arcangelo, et al., 1995; Howell, et al., 1997; Sheldon, et al., 1997; Trommsdorff, et al., 1999).

In contrast, mice deficient in either apoER2 or VLDLR do not have a severe developmental phenotype suggesting that there are some overlapping or cooperative functions between these two receptors during development. Instead these mice show impaired associative learning and mild disruptions to hippocampus area CA1 long-term potentiation (LTP) (Weeber, et al., 2002). The finding of altered synaptic plasticity in the absence of a major developmental phenotype hints at a role for lipoprotein receptor signaling in neuronal function. This is supported by studies manipulating synaptic plasticity by altering lipoprotein receptors. Application of RAP to wild-type hippocampus slices inhibits LTP (Zhuo, et al., 2000). Similar results have been seen with the use of the CR-50 antibody which specifically blocks ligand binding to apoER2 (Nakajima, et al., 1997). The acute involvement of lipoprotein receptors in synaptic plasticity is further demonstrated by the actions of endogenous reelin application in the adult brain.

After development, reelin is still present in the brain, primarily expressed by GABAergic interneurons in the hippocampus and cortex (Campo, et al., 2009). Reelin co-localizes with the postsynaptic density (Beffert, et al., 2005; Roberts, et al., 2005), and this physical presence has sparked intense investigation into the importance of reelin signaling in the adult hippocampus. Application of reelin activates apoER2 and VLDLR and results in a significant enhancement of LTP induction (Weeber, et al., 2002). This enhancement is abolished in the absence of either receptor (Weeber, et al., 2002), indicating a necessity for normal reelin signaling in synaptic plasticity. Reelin signaling is initialized by clustering of both apoER2 and VLDLR followed by tyrosine phosphorylation of Dab-1 by Src family non-receptor tyrosine kinases (SFKs) (Arnaud, et al., 2003; Benhayon, et al., 2003; Bock & Herz, 2003; Hiesberger, et al., 1999; Strasser, et al., 2004). Dab-1 docks to the NPxY sequence of apoER2 and VLDLR through its N-terminal phosphotyrosine-binding (PTB) domain (Howell, et al., 1999). Phosphorylated Dab-1 likely serves as a docking site for the SH2 domain of SFKs, leading to increased Dab-1 activation and alterations in synaptic plasticity (Arnaud, et al., 2003; Bock & Herz, 2003).

The various intracellular pathways that are activated following reelin binding to apoER2 and VLDLR are illustrated in figure 2. It is hypothesized that Reelin signaling via lipoprotein receptors alters synaptic plasticity through signal transduction pathways that modulate N-methyl-D-aspartic acid (NMDA) receptor function. SFKs activated by receptor clustering can phosphorylate tyrosine residues on the NMDA receptor subunits

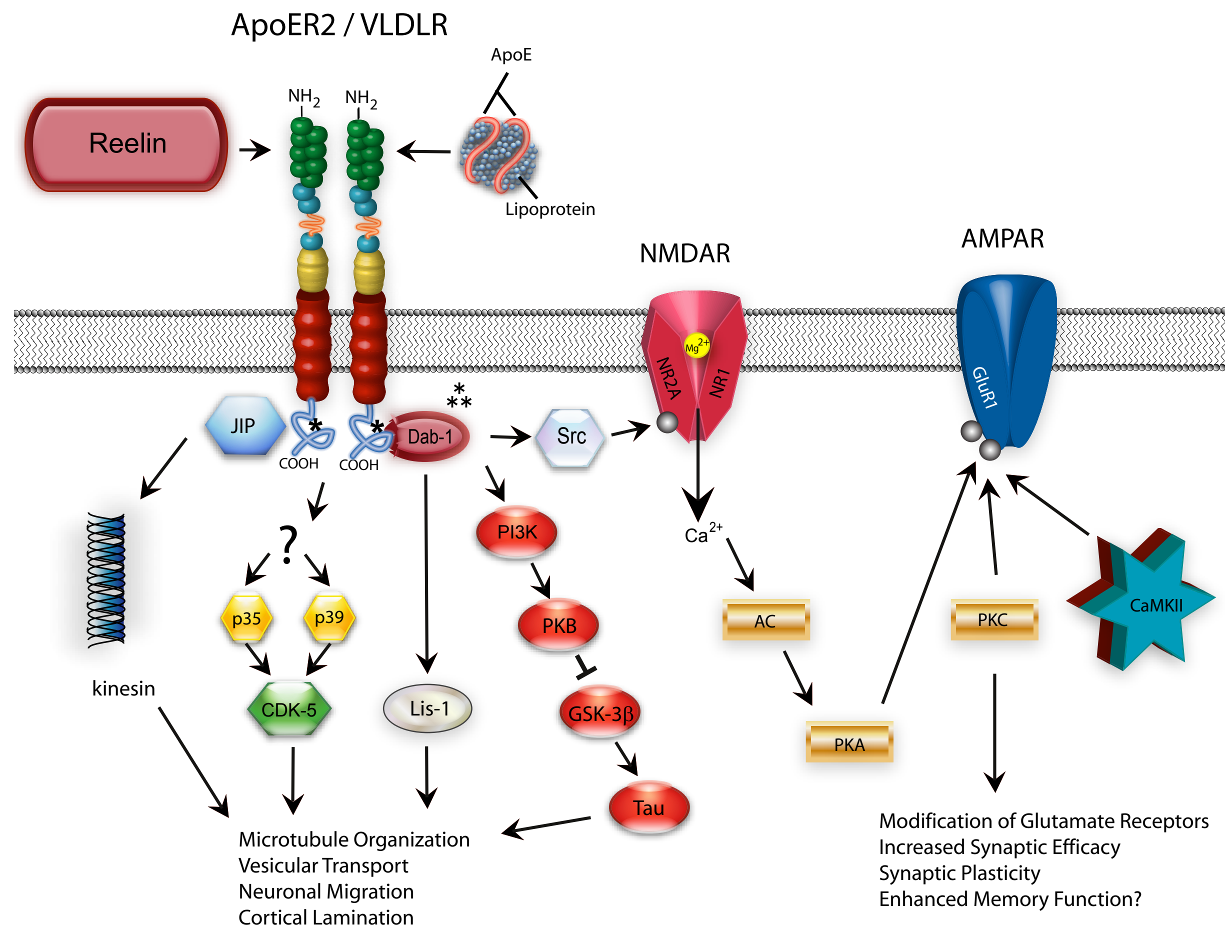


Figure 2: Reelin signaling via apoER2/VLDLR
Reelin interacts with the lipoprotein receptors apoER2 and VLDLR and initiates intracellular signaling. This includes Dab-1, SFKs, PI3K, and CDK-5. The downstream effects of this signaling can potentially modulate cellular processes such as microtubule stability, neuronal migration, glutamate receptor function, and synaptic plasticity.

NR2A or NR2B and enhance NMDA receptor-mediated currents (C. Chen & Leonard, 1996; Kohr & Seeburg, 1996). Reelin-induced enhancement of NMDA receptor-mediated whole cell currents occurs through postsynaptic apoER2 receptors and requires the presence of the splice variant of apoER2 encoded by exon 19 (Beffert, et al., 2005). In addition, reelin contributes to the developmental change in subunit composition of NMDA receptors from NR2B to NR2A. This is dependent on both LDL receptors and downstream SFK activity (Sinagra, et al., 2005). Increased calcium influx due to enhanced NMDA receptor function is likely to activate calcium-calmodulin dependent protein kinase II (CamKII), PKA, and PKC, which are known to be involved in the modulation of ligand-gated ion channels and LTP induction (L. Chen & Huang, 1992; Omkumar, et al., 1996; Tingley, et al., 1997; Zheng, et al., 1998).

In addition, reelin signaling via lipoprotein receptors activates phosphatidylinostiol-3-kinase (PI3K)/protein kinase B (PKB/AKT) pathways. This subsequently inhibits the tau kinase glycogen synthase kinase 3 beta (GSK-3β) (Beffert, et al., 2002; Bock, et al., 2003). Likewise, reelin couples to cyclin-dependent kinase 5 (CDK5), a Ser/Thr kinase whose substrates include tau (Noble, et al., 2003). This links reelin signaling to microtubule stabilization, a hypothesis supported by the finding that animals deficient in either reelin or both apoER2 and VLDLR show hyper-phosphorylation of the microtubule stabilizing protein tau (Hiesberger, et al., 1999). Thus, through these pathways reelin may also alter synaptic plasticity through its influences on cytoskeletal reorganization.

Furthermore, the signaling cascades initiated by reelin can also affect glutamatergic tone by modulation of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors. Exposure to reelin enhances synaptically evoked whole cell AMPA currents. While reelin does not change the levels of AMPA receptor subunit phosphorylation, it does alter total surface levels of these subunits (S. Qiu, Zhao, et al., 2006). Furthermore, application of RAP or two distinct PI3K inhibitors, wortmannin or LY294002, completely blocks reelin-induced enhancement of AMPA receptor currents (S. Qiu, Zhao, et al., 2006). While the mechanism of these changes is unknown, this finding complements the ability of lipoprotein receptors and reelin to alter NMDA receptor activity and synaptic plasticity. This positions the reelin signaling system as an important modulator of synaptic plasticity. With this comes the possibility that other lipoprotein receptor ligands, namely apoE, may be modulating this system as well.

Apolipoprotein E

Apolipoprotein E (apoE) is a 34-kDa secreted glycoprotein with the primary function of mediating cholesterol transport and metabolism via receptor-mediated endocytosis. The greatest expression of apoE is in the liver and the brain. In the periphery, apoE circulates to help transport very low density lipoproteins (VLDLs) that are synthesized by the liver (Wahrle & Holtzman, 2003; Wu, et al., 1998). ApoE, however, does not readily cross the blood brain barrier. In the brain, apoE is synthesized predominantly by astrocytes under basal conditions (Pitas, et al., 1987). ApoE in the brain associates with a pool of lipoproteins that are separate from those in the periphery.

These CSF lipoproteins are similar in size and density to the smaller high density lipoproteins (HDLs) in the periphery (for reviews on CNS lipoproteins, see (Fagan, et al., 2000; Ladu, et al., 2000; Wahrle & Holtzman, 2003).

Human apoE is a 299 amino acid protein. In a lipid-free state, apoE is composed of two independently folded functional domains. The N-terminal domain, as determined by X-ray crystallography, is an elongated four-helix bundle (C. Wilson, et al., 1991). Circular dichroism spectroscopy indicates that the C-terminal domain is also highly α-helical, but its structure is unknown (Segrest, et al., 1992) (figure 3). The receptor-binding domain of apoE is located within the N-terminal domain (residues 136-150) (Siest, et al., 1995) while the major lipid-binding region is within the C-terminal domain (residues 244-272) (Pillot, et al., 1999; Westerlund & Weisgraber, 1993). There are three commonly occurring apoE isoforms in the human population that differ at two amino acid positions: apoE2 (Cys^{112}, Cys^{158}), apoE3 (Cys^{112}, Arg^{158}), and apoE4 (Arg^{112}, Arg^{158}). These isoform variations can alter apoE structure/folding and thus alter associations with lipids and receptor binding (Hatters, et al., 2006).

Each apoE isoform has unique biophysical properties that may contribute to the reported isoform-specific alterations in synaptic plasticity and learning and memory. The residue at position 112 in apoE alters the conformation of the side chain of Arg61 as visualized in x-ray structures of the N-terminal domains of apoE3 and apoE4 (Dong, et al., 1994). The presence of Arg112 in apoE4 results in the side chain of Arg61 being oriented away from the four-helix bundle structure of the N-terminal domain. Conversely, in the presence of cysteine at position 112, the side chain of Arg61 in apoE3

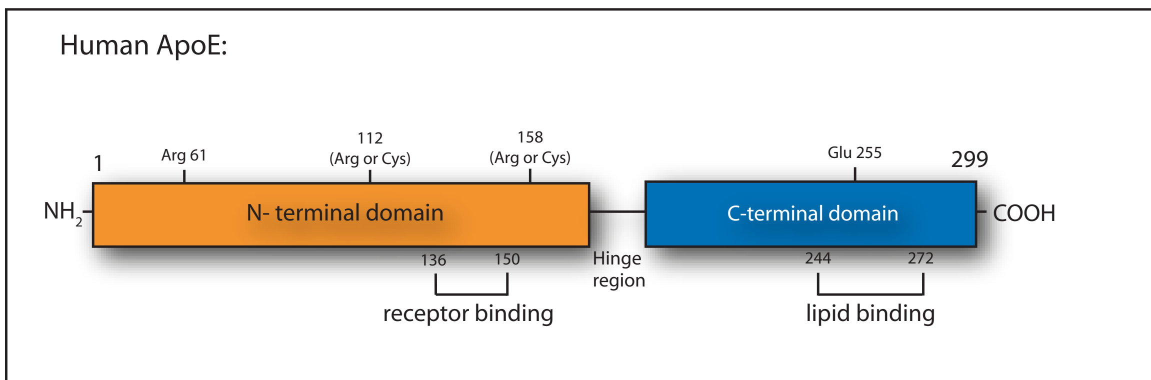


Figure 3: Schematic diagram of human apolipoprotein E (apoE)
Human apoE contains two independently folded domains. Receptor binding occurs within the N-terminal domain, lipid binding within the C-terminal domain. Isoform variation occurs at residues 112 and 158 in the N-terminal domain as illustrated. The residues responsible for domain interaction in apoE4, Arg61 and Glu255, are also indicated (Pillot, et al., 1999; Siest, et al., 1995; Westerlund & Weisgraber, 1993).

is between two helicies. This difference specifically allows for Arg61 in apoE4 to interact with Glu255 in the C-terminal domain (Dong & Weisgraber, 1996).

Humans are unique in their possession of Arg61; apoE in all other species contain threonine at the equivalent position. Thus, apoE in other species functionally behaves like human apoE3 (Hatters, et al., 2006). For example, mouse apoE contains residues equivalent to Arg112 and Glu255 but does not show domain interaction. However, domain interaction can be introduced by the mutation of threonine to arginine at the position equivalent to human Arg61 in mouse apoE (Raffai, et al., 2001). This is sufficient to reproduce some of the properties of apoE4, such as the observed reduction in apoE4 levels versus wild type in hippocampus (Ramaswamy, et al., 2005).

These changes in apoE conformation also alter the stability of the N-terminal domain. The three isoforms of apoE exhibit different thermal and chemical stabilities. The domain interaction of apoE4 correlates with a reduced resistance to thermal and chemical denaturation. Of the three isoforms, apoE2 is the most resistant to this denaturation; apoE3 shows intermediate resistance (Morrow, et al., 2000). This conformational stability and folding of the N-terminal has implications for lipid binding. Variation of the stability of the N-terminal domain may contribute to isoform-specific differences in lipoprotein-binding preferences and other *in vivo* biological functions.

ApoE3 and apoE4 are distributed differently among plasma lipoproteins. Relative to apoE3, there is more apoE4 found in VLDL and LDL fractions than HDL (Gregg, et al., 1986; Weisgraber, 1990). This preference for VLDLs is a result of the domain interaction in apoE4; lipid binding is disrupted by the E255A mutation in apoE4,which

disrupts domain interaction (Dong & Weisgraber, 1996; Saito, et al., 2004; Weisgraber & Mahley, 1996). This association may also underlie association of apoE4 with elevated plasma cholesterol and LDL concentration as well as increased risk for cardiovascular disease (Davignon, et al., 1988; Stengard, et al., 1995; Tiret, et al., 1994; Utermann, et al., 1979; P. W. Wilson, 1995). Unfortunately, lipoproteins in the brain are not as readily isolated as their counterparts in the plasma. It is known, however, that CSF lipoproteins have a size range between those of plasma HDL and LDL. ApoE associates with those lipoproteins that are of the size range of approximately 18-22 nm (Koch, et al., 2001). As apoE receptor affinity is influenced by the type and composition of bound lipid (Kowal, et al., 1990), apoE isoform-dependent variation in lipoprotein affinity may have implications on receptor binding.

The biochemical properties of apoE isoforms also influence receptor binding affinities. ApoE3 and apoE4 have a similar high binding affinity with LDLR. In contrast, binding of apoE2 is approximately 50-100 times weaker (Weisgraber, et al., 1982). The effects of this are seen most readily in the periphery. ApoE2 is associated with type III hyperlipoproteinemia, which is characterized by increased plasma cholesterol and triglyceride levels and premature development of cardiovascular disease (Mahley & Rall, 1995). While the effect of apoE isoform on binding affinity to other lipoprotein receptors is unknown, it is not unreasonable to hypothesize that the above biophysical properties underly isoform-specific alterations to receptor-mediated processes such as neurite outgrowth (Bellosta, et al., 1995; Nathan, et al., 1994) or Aβ clearance (Bales, et al., 1999; Holtzman, et al., 2000; Irizarry, et al., 2004; LaDu, et al., 1994).

ApoE in the mammalian CNS

The transport of cholesterol by apoE, mainly from astrocytes to neurons, helps maintain membranes and myelin sheaths (Ignatius, et al., 1986; Yip, et al., 2005). Yet due to overlapping functions of other apolipoproteins, deletion of the murine *APOE* gene results in viable animals that develop without any gross brain anatomical abnormalities. However, apoE-deficient animals have impaired VLDL metabolism, display elevated plasma cholesterol levels, and spontaneously develop atherosclerosis (Nakashima, et al., 1994). The separation of plasma apoE and associated lipoproteins in the periphery from those in the brain suggests that peripheral cholesterol levels are not directly responsible for apoE-related changes in the brain (Bassett, et al., 2000; Pitas, et al., 1987; Roheim, et al., 1979).

Even though apoE-deficient animals develop without gross brain anatomical deficits, there are numerous studies that illustrate the importance of apoE in maintaining proper neuronal networks. Several *in vitro* studies have illustrated the requirement of apoE-lipoprotein complexes in neurite outgrowth and synapse formation (Fagan, et al., 1996; Mauch, et al., 2001). In addition, mice that are deficient for apoE show an age-dependent depletion in dendritic arborization and synapses in the hippocampus and frontal cortex as visualized by a decrease in microtubule associated protein 2 (MAP2) and synaptophysin (SYN) immunoreactivity. This decrease is accompanied by disruptions to dendritic-associated microtubules, suggesting that these mice are unable to maintain the necessary dendritic structures required for effective synaptic transmission (Masliah, et al., 1995). However, none of these deficiencies were observed in an line of

apoE-deficient animals with a different genetic background, illustrating the complicated relationship between genetic background and apoE-dependent alterations in brain function (Anderson, et al., 1998). Genetic background of mice often changes physiology and behavior. For example, the mice with a C57BL/6J background are more resistant to seizures than those on the 129/SvJ background (McKhann, et al., 2003).

The role of apoE in maintaining neuronal networks can also be influenced by isoform variation and the lipidation state of apoE. Application of apoE in combination with HDL results in isoform-specific increases neurite outgrowth in the CNS-derived cell line GT1-1 trk9; apoE3 has a greater effect than apoE4 (Fagan, et al., 1996). Much of this effect is blocked by application of either RAP or anti-LRP1 antibody, indicating the role of the lipoprotein receptor family in general and the specific lipoprotein receptor LRP1 in mediating the actions of apoE in this specific process (Fagan, et al., 1996). The lipidation state of apoE may also play a role in its function, as apoE/lipoprotein particles secreted by astrocytes have a higher affinity for LDLR than LRP1 (Fryer, et al., 2005). This is contrasted by the behavior of recombinant apoE, apoE-enriched lipoprotein particles, and HDL particles isolated from CSF, which all bind LRP1 more avidly than LDLR (Fagan, et al., 1996; Kowal, et al., 1990).

These findings emphasize the importance of apoE and lipoprotein receptors in neuronal function. However, the role of apoE in human cognition is complicated by the presence of isoform variation. Unique to humans, these three isoforms have the potential to alter apoE-based signaling in the brain, ultimately impacting cognitive processes and the development of neurological disorders.

ApoE and AD

The allelic variation of apoE exerts a differential risk on the development of AD, a finding first reported more than a decade ago (Corder, et al., 1993; Rebeck, et al., 1993). The three human isoforms, apoE2, apoE3, and apoE4, are maintained at different frequencies within human population groups (Corbo & Scacchi, 1999). The apoE3 allele is maintained at an allele frequency of 78% in populations of European descent (Corbo & Scacchi, 1999). ApoE4, with an allele frequency of 14%, is linked to an increased risk of developing sporadic AD in relation to apoE3, as well as decreased age of onset. In contrast, apoE2 expression decreases disease risk in relation to apoE3 (Corder, et al., 1993; Strittmatter, et al., 1993).

Both apoE and apoE receptors have been found to co-localize with amyloid plaque deposits in human AD as well as animal models of AD, including the PDAPP and 5x FAD mice (Arelin, et al., 2002; Poduri, et al., 1994; Shao, et al., 1997). This physical interaction sparked studies into the relationship between apoE, apoE receptors, and Aβ. These studies revealed that there are several lines of evidence beyond physical interaction that suggest a role for apoE and its receptors in the modulation of APP processing and Aβ accumulation.

The amyloid cascade hypothesis of AD centers around excessive formation of Aβ, a product of APP processing. APP undergoes proteolysis by secretase enzymes to either release Aβ, which forms amyloid plaques, or the non-toxic soluble APP (sAPP) peptide. Cleavage by an α-secretase releases sAPPα and precludes Aβ formation as the α-secretase cleave site is within the Aβ region (Turner, et al., 2003). Alternatively, APP is cleaved at

the β-secretase site by β-site APP cleaving enzyme (BACE) releasing sAPPβ. The remaining C-terminal fragment, known as C99, is subsequently cleaved by the γ-secretase/presenilin complex within the transmembrane region to release Aβ peptide and APP intracellular domain (AICD) (Selkoe, 2002b). The magnitude by which this processing can be altered by genetics, age, or other modulatory factors is still being elucidated. At sufficient concentrations, Aβ can form fibrils and oligomers that deposit into plaques within the brain. The longer Aβ42 aggregates and forms fibrils more readily and is more neurotoxic than Aβ40 i*n vitro* (Burdick, et al., 1992; Jarrett, et al., 1993; Snyder, et al., 1994). As previously discussed this Aβ deposition is hypothesized to lead to neuronal injury and cell death by altering synaptic efficacy, inducing inflammatory response in microglia and astrocytes, and inducing changes in cell signaling. This widespread neuronal dysfunction and cell death manifests as dementia in the AD patient.

Research within the context of the Aβ hypothesis is now focused on understanding how APP processing and Aβ deposition is modulated. Importantly, apoE receptors appear to play a role in these processes. For instance, LRP1, LRP1B, apoER2, and the related receptor SORLA interact with APP and can modulate processing to Aβ. For example, LRP1 binds and internalizes sAPPα and interacts with APP via the cytoplasmic adapter protein FE65 (Fiore, et al., 1995; Knauer, et al., 1996). Antagonizing this interaction between APP and LRP1 with RAP in vitro results in an increase in cell surface APP and a decrease in Aβ production (Ulery, et al., 2000). Likewise, apoER2 associates with APP through f-spondin, an extracellular matrix-associated protein (Hoe, Magill, et al., 2006). Expression of f-spondin with apoER2 in

vitro results in reduced Aβ and β-CTFs, an effect that is inhibited by pre-incubation with RAP (Hoe, Magill, et al., 2006). Thus lipoprotein receptors play a dual role in the Aβ hypothesis, either directly interacting with APP or mediating the apoE/Aβ relationship.

ApoE can interact with Aβ through a region in its C-terminal domain and act as a chaperone for Aβ clearance via lipoprotein receptors (Beffert, et al., 1998; Pillot, et al., 1999; Tamamizu-Kato, et al., 2008). The isoform variation of apoE alters this interaction. Both apoE2 and apoE3 bind Aβ and form stable complexes more readily than apoE4 (LaDu, et al., 1994; LaDu, et al., 1997; Pillot, et al., 1997). This interaction has the potential to influence both the clearance of Aβ and the accumulation of Aβ into plaques. ApoE treatment to both neuronal and non-neuronal cell culture also leads to the accumulation of APP c-terminal fragments, suggesting that apoE may modulate the gamma secretase cleavage of APP (Irizarry, et al., 2004).

ApoE4 expression increases amyloid plaque load in AD mouse models (DeMattos, 2004; Holtzman, et al., 2000; Holtzman, et al., 1999), and in human AD (Schmechel, et al., 1993). Crossing the Tg2576 mouse model of AD, which displays a 10-15 fold increase in Aβ, with apoE3 or apoE4 knock-in mice results in a delay in the development of Aβ deposition versus murine apoE expression (Fryer, et al., 2005). Furthermore, this biological effect of apoE correlates with a physiological consequence. For example, neurons cultured from animals expressing apoE4 have a greater susceptibility to Aβ42 -induced neurotoxicity than wild-type, apoE-deficient, apoE2- or apoE3-expressing neurons (Manelli, et al., 2007). Expression of apoE isoform under the endogenous murine promoter also influences susceptibility to Aβ-induced inhibition of

perforant path LTP in a manner that mirrors AD risk (E4>E3>E2) (Trommer, et al., 2005). Crossing the same apoE isoform-expressing animals with the PDAPP transgenic mouse model of AD results in isoform-specific alterations to beta amyloid levels (Bales, et al., 2009). Brain Aβ and amyloid burden are significantly greater in the presence of apoE4 versus apoE2 or apoE3. Additionally, about 90% of the apoE in the brains of the PDAPP animals expressing apoE4 is associated with Aβ, while only about 25% of apoE2 co-localizes with Aβ (Bales, et al., 2009), further emphasizing the role of apoE isoform in this interaction.

ApoE isoform may function apart from interactions with Aβ to affect synaptic integrity and function as well as memory formation. For instance, over-expression of human apoE3 but not apoE4 prevents age related neurodegeneration, namely the loss of SYN-positive presynaptic terminals and MAP2-positive neuronal dendrites in the neocortex and hippocampus that is seen in apoE-deficient mice (Buttini, et al., 1999). Similarly, spatial learning deficits observed in one line of apoE-deficient animals can be reversed by infusion of either human apoE3 or apoE4 (Masliah, et al., 1995). In addition, there may be isoform specific alterations to the stability, expression, or uptake of apoE. In several brain areas, including the hippocampus, apoE4 is detected at lower levels than apoE3 (Ramaswamy, et al., 2005; Riddell, et al., 2008). While all these investigations have yet to elucidate how apoE isoform specifically contributes to synaptic function, there is new evidence that this protein may play a much more complicated role as a signaling ligand in the adult hippocampus.

Receptor interactions and signaling by apoE

While the importance of apoE receptors has been emphasized in several studies, the role of apoE as a signaling molecule has only begun to be considered. Recombinant apoE isoforms differentially affect intracellular calcium signaling and neurotoxicity in cultured hippocampal neurons. ApoE4, but not apoE3, significantly increases neurotoxicity as measured by enhanced lactate dehydrogenase release. This neurotoxicity requires binding with LDLRs, activation of NMDARs, and subsequent increases in intracellular calcium (Z. Qiu, et al., 2004). ApoE also has isoform-specific effects on receptor turnover. ApoE4, but not apoE2 or apoE3, retards apoER2 retroendocytosis, or the resurfacing of the internalized receptor (J Herz, personal communication). Furthermore, apoE blocks reelin-induced increases in intracellular Ca^{2+} in an isoform specific manner, with apoE4 having the greatest effect and apoE2 the least (J Herz, personal communication). These data suggest the presence of specific apoE isoform has the ability to alter receptor availability and subsequent signaling events that control cellular processes.

ApoE has also been implicated in at least three major signal transduction pathways that are known to be involved in synaptic plasticity: extracellular-regulated kinase (ERK), c-jun N-terminal kinase (JNK) and Dab-1/SFKs. In primary neuronal cell cultures, application of either full length apoE or a tandem repeat peptide of the receptor binding domain of apoE significantly enhances phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and Dab-1 while decreasing phosphorylation of c-jun N-

terminal kinase 1/2 (JNK1/2). These effects are mediated by interactions of apoE with LDL receptors (Hoe, et al., 2005).

Interestingly, the apoE-mediated decrease in JNK1/2 phosphorylation requires activity of γ-secretase (Hoe, et al., 2005). Proteolytic processing of APP by γ-secretase is required for the formation of the amyloidogenic Aβ. As previously mentioned, several other transmembrane proteins, including the LDL receptors LRP1, VLDLR and apoER2, Notch, as well as APP also undergo cleavage mediated by γ-secretase (Kopan & Ilagan, 2004). ApoE stimulates γ-secretase dependent lipoprotein receptor processing to varying degrees based on isoform, with apoE2 having the greatest effect and apoE4 the least (Hoe & Rebeck, 2005). The importance of this receptor processing has recently become of increased interest due to the pervasiveness of soluble apoE receptors in the mammalian brain and the role these soluble receptors play in cell signaling (for review see (Rebeck, et al., 2006)). For instance, soluble apoER2 binds membrane-bound apoER2 and VLDLR and blocks subsequent Reelin-induced signaling through these receptors in primary neuronal culture (Koch, et al., 2002) lending evidence to the hypothesis that apoE is involved in neuronal processes that extend beyond the transport of cholesterol.

The apoE targeted replacement mouse

Many of the previously mentioned findings are the result of *in vitro* studies in cultured neurons. While these results are significant, interpretations about *in vivo* apoE function in animals or humans are limited. Attempts to use apoE isoform transgenic animals have presented their own set of caveats, from varying expression levels between

isoforms to complications due to murine apoE. Performing these studies in an animal that expressed human apoE isoforms under the control of the endogenous murine promoter, such as the apoE targeted replacement (apoE TR) mouse developed in Dr. Nobuyo Maeda's laboratory, would greatly advance our understanding of the role of apoE in memory formation and synaptic function (Sullivan, et al., 1997).

The apoE2, apoE3, and apoE4 TR animals express each of the human apoE isoforms under the control of the endogenous murine promoter thus preserving the natural expression patterns between isoforms (Sullivan, et al., 2004; Sullivan, et al., 1997). While there have been no developmental alterations reported, there are significant differences in synaptic plasticity and behavior between isoform expressing animals. ApoE expression in apoE TR mice alters perforant path LTP in an isoform specific manner. ApoE3 TR and wild-type animals have the highest amount of LTP induction followed by apoE2 TR and apoE-deficient animals, and finally apoE4 TR animals with the least (Trommer, et al., 2005). Conversely, in a separate but equivalent line of apoE TR animals, LTP induction measured in hippocampus area CA1 is increased in apoE4 TR animals over wild-type animals (Kitamura, et al., 2004). This enhancement in CA1 LTP with apoE4 expression has been observed again in the apoE TR line developed by Sullivan *et al.*, and apoE4 expression also blocks further enhancement of LTP by reelin (J Herz, personal communication).

ApoE isoform expression in the apoE TR animals also alters learning and memory behavior. Female apoE4 TR mice have impairments in spatial memory retention in comparison to apoE3 TR, apoE-deficient, and murine apoE-expressing animals

(Grootendorst, et al., 2005). These deficits in the apoE4 TR mice persist with age (Bour, et al., 2008). However, apoE2 TR animals have not been tested along side apoE3 TR and apoE4 TR animals under the same conditions. Another study of these animals found no alterations to spatial memory. Instead, apoE4 TR mice are impaired in anxiogenic tasks such as passive avoidance, light-dark, and elevated plus maze (Villasana, et al., 2006). Likewise, conflicting reports from previous behavioral studies also plague the literature involving apoE-deficient animals. Spatial learning in the apoE-deficient mouse is particularly variable. In several studies, spatial memory is equivalent between apoE-deficient and murine apoE-expressing animals (Anderson, et al., 1998; Anderson & Higgins, 1997; Grootendorst, et al., 2005; Raber, et al., 2000). In others, spatial learning is impaired in the absence of apoE (Champagne, et al., 2002; Krzywkowski, et al., 1999; Masliah, et al., 1997; Oitzl, et al., 1997; Veinbergs, et al., 1999), emphasizing the sensitivity of behavioral tests to genetic background, housing conditions, experimental designs, and other extrinsic factors (see table 4). Thus, the field would benefit greatly from single study comparing apoE-deficient animals with all apoE TR animals at various ages.

Summary

The main pathological hallmark and post-mortem diagnostic criterion for Alzheimer's disease is the presence of extracellular plaques composed of Aβ. Processed from the transmembrane protein APP, Aβ has been characterized as the main villain in the

development of AD. Yet decades of research have shown that Aβ is not the only player in this neurodegeneration and attention has turned to other AD risk factors.

In the context of AD research, a great deal of attention has been given to the LDLR family because of another ligand for these receptors—apolipoprotein E. The three human isoform variants of apoE have been linked to altered risk of developing AD. The molecular mechanisms for apoE isoform-dependent contributions to the etiology of AD are unclear. However, there is mounting evidence that apoE isoforms can differentially alter neuronal signaling and synaptic plasticity. These studies lend support to the idea that apoE isoforms are involved in both the pathogenesis and progression of AD and also contribute to neuronal function that can influence cognitive ability.

Studies to date illustrate the complicated interplay between apoE isoform, synaptic plasticity, and cognition. Deciphering the mechanism by which apoE isoform alters AD risk is further complicated by the pathological changes associated with the disease. Therefore, the only way to fully understand the involvement of this risk factor in the development of AD is to understand its normal function in both synaptic plasticity and learning and memory. Fortunately, characteristics of hippocampus area CA1 make it ideally suited for studying these effects.

Hypothesis and specific aims

Signaling through lipoprotein receptors is necessary for normal synaptic functioning in the adult hippocampus. The capacity of apoE to signal through these receptors is one of the many unanswered questions surrounding this receptor system. The

potential interaction between apoE and ligands for specific lipoprotein receptors may alter signal transduction cascades essential to learning and memory processes. Without an understanding of the normal role of apoE isoforms in synaptic plasticity, the mechanism by which apoE isoform impacts Alzheimer's disease risk will be nearly impossible to resolve.

The studies proposed here are the first to investigate the effect of both acute apoE isoform application and chronic apoE isoform expression on synaptic plasticity in area CA1 of the hippocampus. This project capitalizes on the currently available apoE TR mice as well as isoforms of recombinant human apoE to test the hypothesis that apoE acts as an isoform-specific signaling ligand for lipoprotein receptors in the adult hippocampus to modulate neuronal synaptic plasticity. Additionally, this study uses the apoE TR mice to test the effects of human apoE isoform on hippocampus-dependent learning and memory behavior.

Hypothesis 1: ApoE acts in an isoform-specific manner to modulate synaptic function in the adult hippocampus

Specific aim 1: Determine the effect of apoE isoform on synaptic plasticity of the CA1 dendritic field

Specific aim 2: Determine the synaptic mechanisms involved in apoE isoform-dependent modulation of synaptic plasticity.

Efforts to effectively determine the mechanism by which apoE isoform alters AD risk will be aided by an understanding of the actions of apoE isoforms in the non-AD brain. The current literature is limited in this respect and is complicated by a variety of different techniques, apoE protein sources, and brain regions investigated which has

created a fractured, incomplete model and makes direct comparisons impossible. Therefore, this project was designed to address these confounds by 1) utilizing both acute apoE treatment with recombinant human apoE isoforms and chronic apoE expression in the apoE TR mice; 2) focusing on hippocampus area CA1, which is extremely well-characterized, important in learning and memory, and enriched in LDLRs; and 3) comparing all three human apoE isoforms to each other, as well as apoE deficiency to murine apoE in order to eliminate inter-experimental variables.

Chapters II and III respectively describe the effects of acute and chronic human apoE isoform exposure on synaptic transmission, short- and long-term plasticity in area CA1 of the hippocampus. Further electrophysiological techniques confirmed the role of apoE as a signaling molecule in the adult hippocampus. Finally, combination of electrophysiological and biochemical techniques were used to investigate the mechanism by which apoE isoform alters synaptic plasticity.

Hypothesis 2: Human apoE isoform expression differentially affects learning and memory in the targeted replacement mouse.

Specific aim 3: Determine the effect of apoE isoforms on learning and memory behavior.

The apoE TR mouse is a valuable tool in the elucidation of the role of apoE isoforms in normal learning and memory and in the understanding of AD development in terms of cognitive ability. However, it is necessary to establish a baseline behavioral profile for all three human isoforms prior to further manipulations to understand AD etiology, such as crossing the apoE TR animals with other AD mouse models. As

described in Chapter IV, this study investigated the behavioral consequences apoE isoform in these targeted replacement animals. Using young adult (3-5 months) animals, this study reports changes in performance of learning and memory behavior tasks, focusing on motor learning, working memory, spatial memory, and associative learning.

CHAPTER II

ISOFORM-DEPENDENT CHANGES IN HIPPOCAMPUS SYNAPTIC FUNCTION WITH ACUTE APOE ISOFORM EXPOSURE

Introduction

Located within the medial temporal lobe, the hippocampus is essential for proper memory formation (Amaral & Lavenex, 2006; Eichenbaum, 1999). It receives both direct and indirect input from all sensory areas of the cortex as well as modulatory input from various areas of the brainstem. The hippocampus proper has three major pathways (figure 4): 1) the perforant pathway that projects from the entorhinal cortex to the granule cells of the dentate gyrus; 2) the mossy fiber pathway composed of granule cell axons that project to pyramidal cells in area CA3 of the hippocampus; and 3) the Schaffer collateral pathway that connects CA3 pyramidal cells with those in area CA1.

The pathways of the hippocampus are very sensitive to the stimulation that they have received. Brief, high-frequency stimulation will increase the amplitude of excitatory postsynaptic potentials specifically in the target neurons of the stimulated pathway (Bliss & Lomo, 1970). This enhancement, called long-term potentiation (LTP), can last for hours in the hippocampal slice and for days to weeks in the intact animal (Bliss & Lomo, 1970). LTP has been intensely studied since its discovery and has been loosely defined as a model of learning and memory, albeit with certain caveats. While many genetic or pharmaceutical manipulations that enhance LTP also enhance learning and memory function, there are a myriad of examples that show the opposite relationship or no

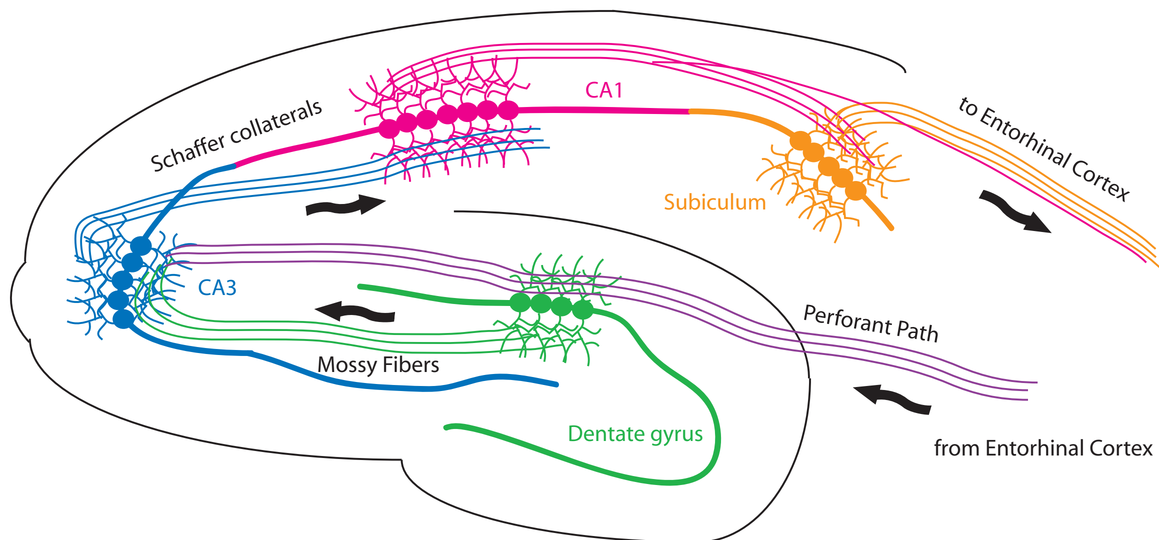


Figure 4: Major pathways in the hippocampal circuit
The hippocampus proper consists of a primarily uni-directional network. Input from the entorhinal cortex forms connections with the dentate gyrus and CA3 pyramidal neurons via the perforant path. The mossy fiber pathway consists of the dentate gyrus granule cells that extend to CA3 pyramidal cells. CA3 pyramidal neuron axons subsequently extend to CA1 forming the Schaffer collaterals. CA1 neurons also received input from the perforant path and subsequently send axons to the subiculum; these neurons then in turn project back to the entorhinal cortex.

relationship at all (Allen, et al., 2000; Gu, et al., 2002; Jun, et al., 1998; Meiri, et al., 1998; Migaud, et al., 1998; Minichiello, et al., 1999; Pavlov, et al., 2002; Walther, et al., 1998). This is likely due to the fact that while plasticity of neuronal communication underlies learning and memory, we know that memories are not stored in the hippocampus. Thus changes in artificially induced plasticity do not directly relate to plasticity in other areas of the brain. In addition, there are other forms of plasticity in the hippocampus, including long-term depression (LTD), which have also been implicated as a mechanism of memory formation. Despite these shortcomings, the study of LTP in the different areas of the hippocampus has been valuable in understanding the molecular mechanism underlying synaptic plasticity.

The CA areas of the hippocampus are so named due to the historical description of hippocampus anatomy as a "ram's horn", or *Cornu Ammonis* (Amaral & Lavenex, 2006). While LTP is possible in all pathways of the hippocampus, this project focuses on changes within the population of pyramidal cells of area CA1. Along with the entorhinal cortex, area CA1 is one of the first brain areas to show AD pathology (Braak & Braak, 1998). In addition, the basic mechanism of synaptic plasticity in area CA1 is well characterized and the lipoprotein receptors are known to modulate area CA1 synaptic function.

The axons that compose the Schaffer collaterals arise from cells in area CA3, synapse on the dendrites from cells in area CA1, and use glutamate as their neurotransmitter. During normal synaptic transmission, glutamate is released from the presynaptic terminal and binds to glutamate receptors on the postsynaptic terminal. While

the glutamate binds to both NMDA and non-NMDA (AMPA) receptors, only the AMPA receptors are activated and allow sodium and potassium ions to flow across the cell membrane. The NMDA receptors remained blocked by magnesium ions in the channel pore at resting membrane potential (Waxham, 2003).

The induction of LTP at these synapses requires concurrent presynaptic release of glutamate and postsynaptic depolarization. The cooperative firing of several axons depolarizes the postsynaptic cell and releases the magnesium block from the NMDA receptor channel. Once this block is released, calcium can flow into the postsynaptic cell and trigger second messenger systems that lead to changes within the postsynaptic cell and LTP (for review, see (Dingledine, et al., 1999)).

The intracellular signaling events initiated by calcium influx are modulated by a variety of other signaling pathways that can increase, decrease, or occlude LTP induction. Most relevant to this study is the modulation of LTP induction by reelin signaling. Reelin binding to its two partners, the lipoprotein receptors apoER2 and VLDLR, initiates signaling cascades that ultimately lead to the enhancement of LTP (Weeber, et al., 2002). Reelin also induces trafficking of AMPA receptors and reduces the number of silent synapses, thereby increasing synaptic efficacy (S. Qiu, Zhao, et al., 2006).

The interplay between the NMDA receptor and lipoprotein receptors is also vital to the study of the role of apoE isoforms in synaptic plasticity. The NMDA receptor, specifically the NR1 subunit, interacts with apoER2 via its extracellular domain; the NR2 subunit links to apoER2 via binding to PSD95 (Beffert, et al., 2005; Hoe, Pocivavsek, Chakraborty, et al., 2006). This places apoER2 in close proximity to the NMDA receptor

and means that signaling by ligands such as apoE through this receptor could have effects on NMDA receptor function.

Both NR2A and NR2B can be tyrosine phosphorylated by the SFKs src and fyn (Cheung & Gurd, 2001), which regulates receptor activity. SFK-dependent tyrosine phosphorylation increases during LTP, suggesting that it is intimately linked to synaptic plasticity and neuronal functioning (Lu, et al., 1998). Reelin signaling, as previously discussed, can modulate the signaling pathways that lead to tyrosine phosphorylation of the NMDA receptor, suggesting the hypothesis that apoE binding to apoER2 and VLDLR may either alter signaling by reelin or similarly initiate signaling events that alter NMDA receptor phosphorylation and synaptic plasticity.

There is also mounting evidence that apoE may act as a signaling molecule to otherwise alter synaptic transmission. For instance, the ERK1/2 signaling pathway, activated by calcium influx through NMDA receptors, plays a key role in synaptic plasticity and cell survival (Sweatt, 2004; Thomas & Huganir, 2004). ApoE can activate ERK1/2 in an isoform dependent manner in neuronal cultures, a process that requires calcium influx through the NMDA receptor. In addition, apoE decreases activation of JNK1/2 and increases PI3K activation via Dab-1 (Hoe, et al., 2005). This suggests that apoE may act as an isoform-specific signaling molecule in the adult brain.

In this study, I explore the ability of apoE isoforms to modulate CA1 synaptic plasticity in the adult mouse. I use field recording techniques to test acute effects of recombinant human apoE isoforms on synaptic plasticity. I also use both

electrophysiological and biochemical techniques to investigate the potential mechanism by which apoE isoform alters synaptic plasticity.

Methods

Animal maintenance

ApoE knock-out animals were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were on a fully backcrossed C57BL/6J background and bred in a homozygous fashion for experimentation. Animals were housed in a standard 12 hour light cycle and bred and maintained in accordance with the Vanderbilt University Institutional Animal Care and Use Committee protocol. Animals were given *ad libitum* access to standard mouse chow (LabDiet, PMI Nutrition International) and water.

Electrophysiology

Hippocampus slices were prepared from 3- to 5-month old mice for field electrophysiology. The animals were sacrificed by cervical dislocation and the brain was rapidly removed and placed in ice-cold high sucrose cutting saline solution containing (in mM) 110 sucrose, 60 NaCl, 3 KCl, 28 $NaHCO_3$, 1.25 NaH_2PO_4, 5 glucose, 0.6 ascorbate, 7 $MgCl_2$, and 0.5 $CaCl_2$ with continuous perfusion of 95% O2/5% CO2 Horizontal 400µm sections were cut in high sucrose cutting solution using a vibratome. Slices were maintained in cold, oxygenated cutting solution until dissection of the hippocampus from the surrounding tissue.

After dissection, the hippocampus slices were transferred to room temperature cutting solution diluted 1:1 with artificial cerebral spinal fluid (ACSF). ACSF contains, in mM, 125 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 25 glucose, 1 $MgCl_2$, and 2 $CaCl_2$. Slices were maintained in this solution with constant 95% O_2/5% CO_2 perfusion for 10 min before transferring to the interface brain slice recording chamber (Fine Science Tools, San Francisco, CA).

The recording chamber was maintained at 30°±0.5°C with a laminar ACSF flow rate of approximately 1 mL/min. Field excitatory postsynaptic potentials (fEPSPs) were recorded from area CA1 *stratum radiatum* via glass micropipettes pulled to an approximate 1 μm tip diameter (1-4 MΩ) and filled with ACSF. Responses were generated by stimulation of fibers arising from the CA3 region. Stimulating electrodes consisting of formvar-coated nichrome wire delivered biphasic stimulus pulses (1-15 V, 100 μsec duration, 0.05 Hz). Delivery of stimulation, controlled by pClamp 9.0 software (Axon Instruments, Forster City, CA), was via the Digidata 1322A interface (Axon Instruments) and a stimulus isolator (model 2200, A-M Systems, Sequim, WA). Signals were amplified using a differential amplifier (model 1800, A-M Systems), filtered at 1 kHz and digitized at 10 kHz. For all experiments, baseline stimulus intensity was set at the level that elicited 40-50% of the maximum fEPSP response as determined from the input-output curve.

Paired-pulse facilitation was induced by delivery of two stimuli in close temporal proximity. Intervals between stimuli began at 20 ms and increased to 300 ms in 20 ms

intervals. Paired-pulse facilitation was calculated by the percent facilitation of fEPSP slope of second response from first response.

Long-term potentiation (LTP) was induced by theta-burst protocol. Theta-burst LTP protocol consisted of five trains of 10 bursts at a 5Hz frequency with each burst consisting of 4 stimulations delivered at 100Hz and an inter-train interval of 20 seconds. NMDAR field potentials were isolated by incubating slices on the rig in ACSF containing 0.1 mM Mg^{2+} for 30 minutes followed by addition of 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 25 μM picrotoxin. After 20 minutes of stabilization, slices were treated with 100 nM of recombinant human apoE isoforms (Calbiochem).

Electrophysiological data was also analyzed using one-way ANOVA with Bonferroni's post hoc tests. Significance was set at $p<0.05$ for all tests.

Biochemistry

Slices were obtained from apoE-deficient mice in an identical fashion as for electrophysiology (see above). Slices (n=3-4 per treatment) were incubated in ACSF at 30°C for 1 hour prior to the addition of 100 nM recombinant human apoE2, apoE3 or apoE4 (Calbiochem) for 40 minutes. Slices were then flash frozen on dry ice and CA1 was dissected.

NMDAR subunit phosphorylation - Pooled tissue was sonicated in modified RIPA buffer (Tris/HCl pH 7.4, 2 mM EDTA, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X100, 1X phosphatase inhibitors I and II (Sigma), and 1X

complete protease inhibitors (Sigma). Protein concentrations were determined by BCA Protein Assay. A total of 400 μg of protein lysate was used to immunoprecipitate either NMDA-NR2A (NMDAε1, Santa Cruz Biotechnology) or NMDA-NR2B (NMDAε2, Santa Cruz Biotechnology) overnight at 4°C with agitation. Protein A/G magnetic beads (New England BioLabs) were added to each reaction (25 μl bead slurry/reaction) and samples were incubated for 2 hours at 4°C with agitation. Following three wash cycles, the protein was eluted with 1X Laemmli sample buffer separated by SDS-page on 4-15% Tris-HCl gradient gels (BioRad) and transferred to PVDF membranes. Membranes were probed with goat anti-NMDAε1, rabbit anti-NMDA, or mouse anti-pTyr (PY99) (Santa Cruz Biotechnology) in 2% BSA-TBST. Membranes were developed using HRP-conjugated secondary antibodies and enhanced chemiluminescence.

ERK/JNK activation – Pooled tissue was homogenized in NP-40 lysis buffer containing (in mM) 50 Tris-HCl pH 8.0, 150 NaCl, 1 EDTA, 1 PMSF, 1 Na_3VO_4, 1 NaF, 1 μg/mL each of aprotinin, leupeptin and pepstatin, and 1% NP-40. Protein concentration was determined by Bradford Assay (BioRad). Ten μg of protein was resolved on 10% SDS-PAGE. Membranes were probed with rabbit anti-ERK1/2, anti-ERK1/2 $pTpY^{185/187}$, anti-JNK1/2, anti-JNK1/2 $pTpY^{183/185}$ (Invitrogen) diluted in 0.24% I-block. Membranes were developed using HRP-conjugated secondary antibodies and enhanced chemiluminescence.

Optical density of immunoreactivity was quantified by densitometry using Image J software (NIH).

Results

Acute exposure to apoE isoforms alters LTP induction

ApoE-deficient animals develop normally without gross pathological changes to brain organization, suggesting that other lipoproteins are able to compensate for the lack of apoE during development. In the hippocampus, synaptic transmission is also unaffected by the lack of apoE: the relationship between fiber volley amplitude and field excitatory postsynaptic potential (fEPSP) slope is equivalent between apoE-deficient and murine apoE-expressing animals (data not shown). This ensures that there are not major synaptic transmission deficiencies in the apoE-deficient animals that would inhibit study in the effect of apoE isoforms.

To isolate the effects of apoE isoforms on synaptic plasticity, apoE-deficient slices were perfused with 100 nM of recombinant human apoE2, apoE3, or apoE4 (rhapoE) for five minutes prior to experimentation. Short term plasticity was measured with paired-pulse facilitation. Two stimuli were delivered in close temporal proximity, increasing in 20 ms intervals from 20 to 300 ms. The fEPSP slope elicited for both stimulations was measured and the percent increase of the second response calculated. ApoE-deficient slices in the absence of rhapoE displayed the typical percent facilitation (figure 5A). Perfusion with rhapoE2, rhapoE3, or rhapoE4 did not change this relationship (figure 5A).

High frequency stimulation of Schaeffer collateral synapses using a standard theta burst stimulation protocol induces robust potentiation (Larson, et al., 1986). Long-term

potentiation was successfully induced in apoE-deficient mice using five trains of theta burst stimulation. To test the hypothesis that apoE isoforms differentially modulate synaptic activity, apoE-deficient hippocampal slices were perfused with 100 nM rhapoE isoforms beginning five minutes prior to the start of baseline recording and continuing for the duration of the experiment. Following theta burst stimulation there was an isoform-dependent change in synaptic plasticity (figure 5B). Quantification of the average fEPSP slope of the last 20 minutes of recording shows that treatment with rhapoE4 significantly increased LTP induction over rhapoE2, respectively (figure 5C, ANOVA, p=0.0112).

Effect of apoE isoform exposure on NMDAR function and phosphorylation

The NMDA receptor is intimately associated with many forms of synaptic plasticity and long-term potentiation, including theta-burst-induced LTP (Larson & Lynch, 1988). In addition, signal transduction via apoE receptors is linked to NMDA receptor maturation (S. Qiu & Weeber, 2007; Sinagra, et al., 2005), increased NMDA receptor currents (S. Qiu, Zhao, et al., 2006), and activation of signaling pathways that involve NMDA receptor function (Beffert, et al., 2005; Hoe, Pocivavsek, Chakraborty, et al., 2006; Weeber, et al., 2002). These results lead to the hypothesis that the observed alterations in LTP may be due to apoE isoform-specific changes in NMDA receptor function. Thus, I next investigated the effect of apoE isoform on CA1 NMDA receptor function.

NMDA receptor field potentials were isolated first incubating slices in ACSF with reduced Mg^{2+} (0.1 mM) for 30 minutes followed by application of 20 µM of the AMPA

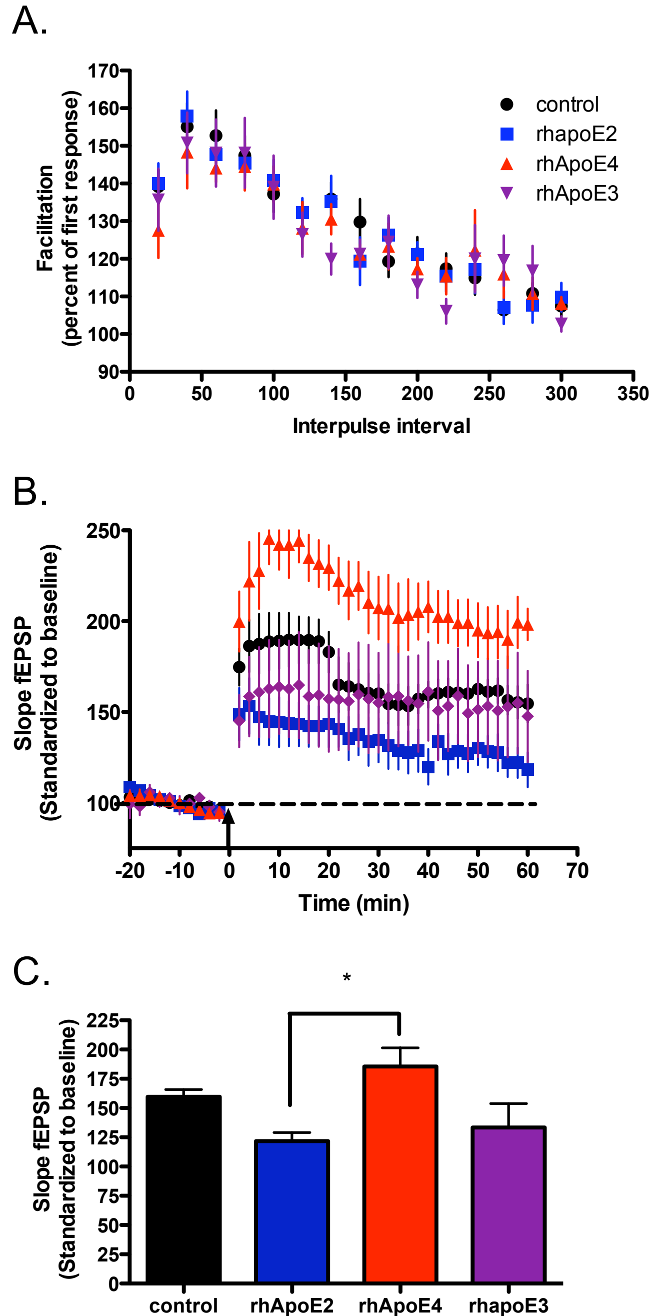


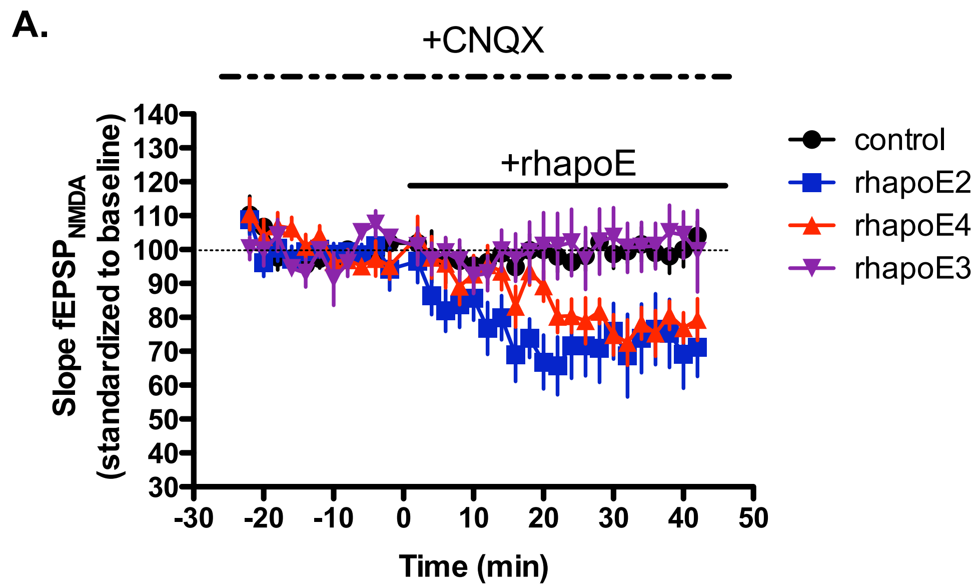
Figure 5: Acute apoE isoform exposure alters LTP in apoE-deficient hippocampus slices

ApoE-deficient slices treated with 100 nM of rhapoE beginning five minutes prior to recording and continuing throughout the duration of the experiment. A. Paired-pulse facilitation as measured by percent facilitation of second response. Second stimuli delivered at 20 ms intervals from 20 to 300 ms from first stimuli. B. LTP induced by 5 trains of theta burst stimulation (arrow). Slope fEPSP standardized to first 20 minutes of recording. C. Average slope fEPSP of last 20 minutes of recording. control = black, n=8; rhapoE2 = blue, n=8; rhapoE3 = purple, n=7; rhapoE4 = red, n=6. Data expressed as mean ± SEM.*p<0.05, ANOVA with Bonferroni's posttest.

receptor antagonist CNQX for 20 minutes. Slices were subsequently treated with 100nM rhapoE2, rhapoE3 or rhapoE4 for 40 minutes, with the NMDAR-mediate field potentials monitored throughout. While I hypothesized that the enhanced LTP seen in the presence of apoE4 was related to increased NMDA receptor currents similar to the effect of reelin application, addition of either recombinant apoE2 or apoE4 significantly reduced NMDA field potentials from control levels (figure 6, p=0.0015). Interestingly, there was no effect on NMDAR field potentials with the application of rhapoE3.

Tyrosine phosphorylation of NMDA-receptor subunits has been shown to influence NMDA receptor trafficking and assembly (Ferrani-Kile & Leslie, 2005; Kohr & Seeburg, 1996). NMDA receptor currents are potentiated by increases activity of protein tyrosine kinases (PTKs) and reduced by decreasing PTK activity (Y. T. Wang & Salter, 1994; Y. T. Wang, et al., 1996) This tyrosine phosphorylation, mediated by SFKs such as fyn, plays an integral role in hippocampal synaptic plasticity and LTP (Ho, et al., 2004; Sala & Sheng, 1999; Zheng, et al., 1998). With the identification of reduced NMDA receptor-dependent field potentials with rhapoE2 and rhapoE4, I hypothesized that apoE may be altering the state of NMDA-receptor subunit tyrosine phosphorylation by decreasing src-dependent phosphorylation, increasing phosphatase activity, or both.

Hippocampus slices from apoE-deficient animals were treated with 100 nM rhapoE isoforms for 40 minutes. These slices were flash frozen and the CA1 region was dissected. We then immunoprecipitated the NMDA receptor subunits NR2A and NR2B from these pooled CA1 samples then probed for tyrosine phosphorylation. There was a trend towards reduced tyrosine phosphorylation of NR2A with application of rhapoE2,



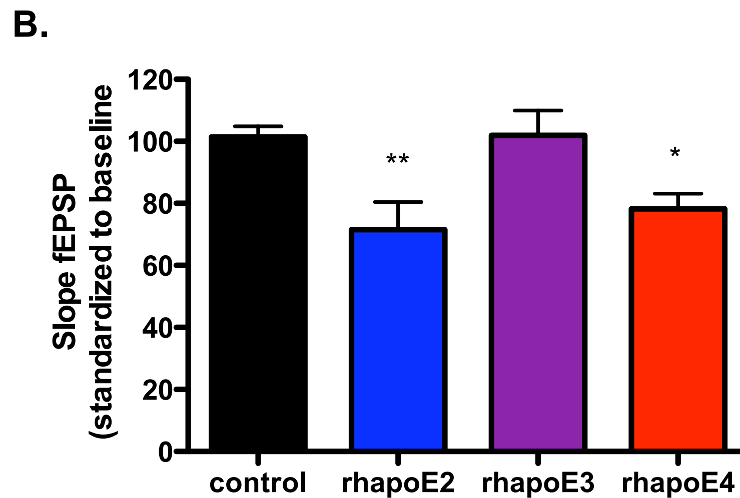


Figure 6: Application of rhapoE alters NMDAR-mediated field potentials
A. NMDAR-mediated field potentials isolated by application of 20 µM CNQX (dashed line). Field potentials measured in presence of 100 nM rhapoE (solid line), standardized to first 20 minutes of recording. B. Average slope fEPSP of last 20 minutes of recording. control = black, n=8; rhapoE2 = blue, n=8; rhapoE3 = purple, n=7; rhapoE4 = red, n=6. Data expressed as mean ± SEM. *$p<0.05$, **$p<0.01$ versus control, ANOVA with Bonferroni's posttest.

rhapoE3, or rhapoE4; control was significantly different from rhapoE4 (figure 7A, ANOVA p=0.0216). Levels of NR2A were unchanged with apoE isoform application (figure 7B, ANOVA p=0.1699). There were no significant differences in the ratio of pNR2A to NR2A between any of the conditions (figure 7C, ANOVA p=0.8550). Also unchanged were levels of pNR2B and NR2B (figure 7D,E ANOVA pNR2B p=0.3287, NR2B p=0.9079). Similarly, there were no significant differences in pNR2B to NR2B ratios with apoE isoform exposure (figure 7F, ANOVA p=0.2655).

Effect of apoE isoform exposure on signal transduction

ApoE has been previously shown in neuronal cell culture to have isoform specific effects on signal transduction pathways crucial to synaptic plasticity such as ERK and JNK (Hoe, et al., 2005). As those effects were blocked by LDL receptor inhibitors and the NMDA receptor antagonist MK-801, we hypothesized that these signaling pathways may also be involved in the isoform-specific alterations in CA1 LTP. To test this possibility, apoE-deficient hippocampus slices were isolated and incubated with 100 nM rhapoE for 40 minutes before flash freezing on dry ice and rapidly dissecting out CA1. Tissue samples were probed by western blot analysis for pERK, pJNK, ERK and JNK.

ApoE isoform application did not significantly alter total pERK or ERK levels (figure 8A,B ANOVA pERK p=0.5100, ERK p=0.8693). There were no significant differences in the pERK/ERK ratio (figure 8C, ANOVA p=0.7154). Furthermore, acute apoE isoform application did not significantly alter either JNK or pJNK levels, or pJNK/JNK ratio (figure 8D-F, ANOVA pJNK p=0.7900, JNK p=0.7627, pJNK/JNK p=0.8414).

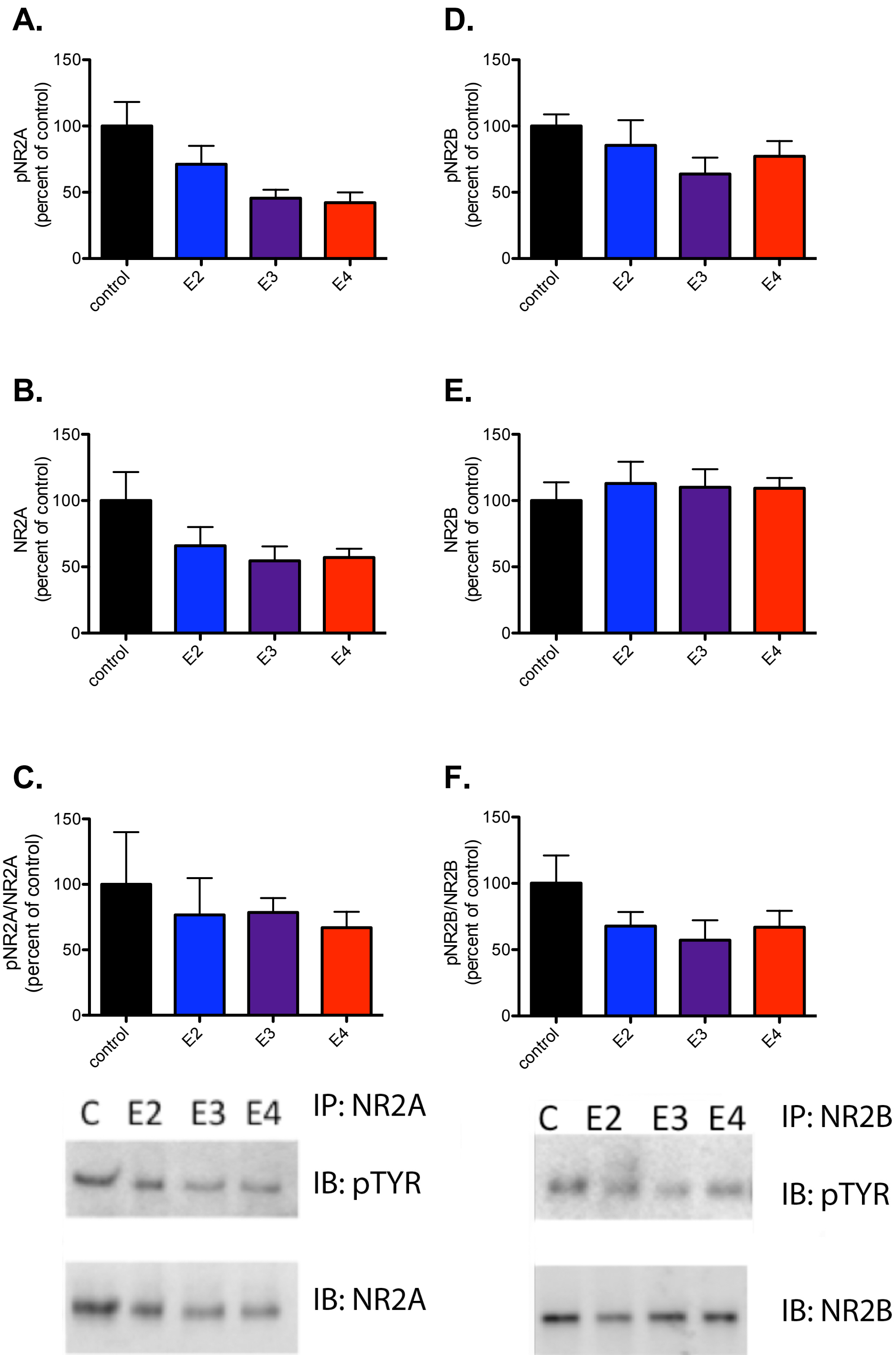


Figure 7: Effect of acute apoE isoform exposure on NR2A and NR2B tyrosine phosphorylation

ApoE-deficient slices treated with 100 nM rhapoE isoforms.

Immunoreactivity of A) pTyr and B) NR2A following immunoprecipitation by NR2A. C) Ratio of pNR2A to NR2A

Immunoreactivity of D) pTyr and E) NR2B following immunoprecipitation by NR2B. F) Ratio of pNR2B to NR2B. control= C, black (n=6), rhapoE2 = E2, blue (n=7), rhapoE3 = E3, purple (n=5), rhapoE4 = E4, red (n=5). Data expressed as mean ± SEM.

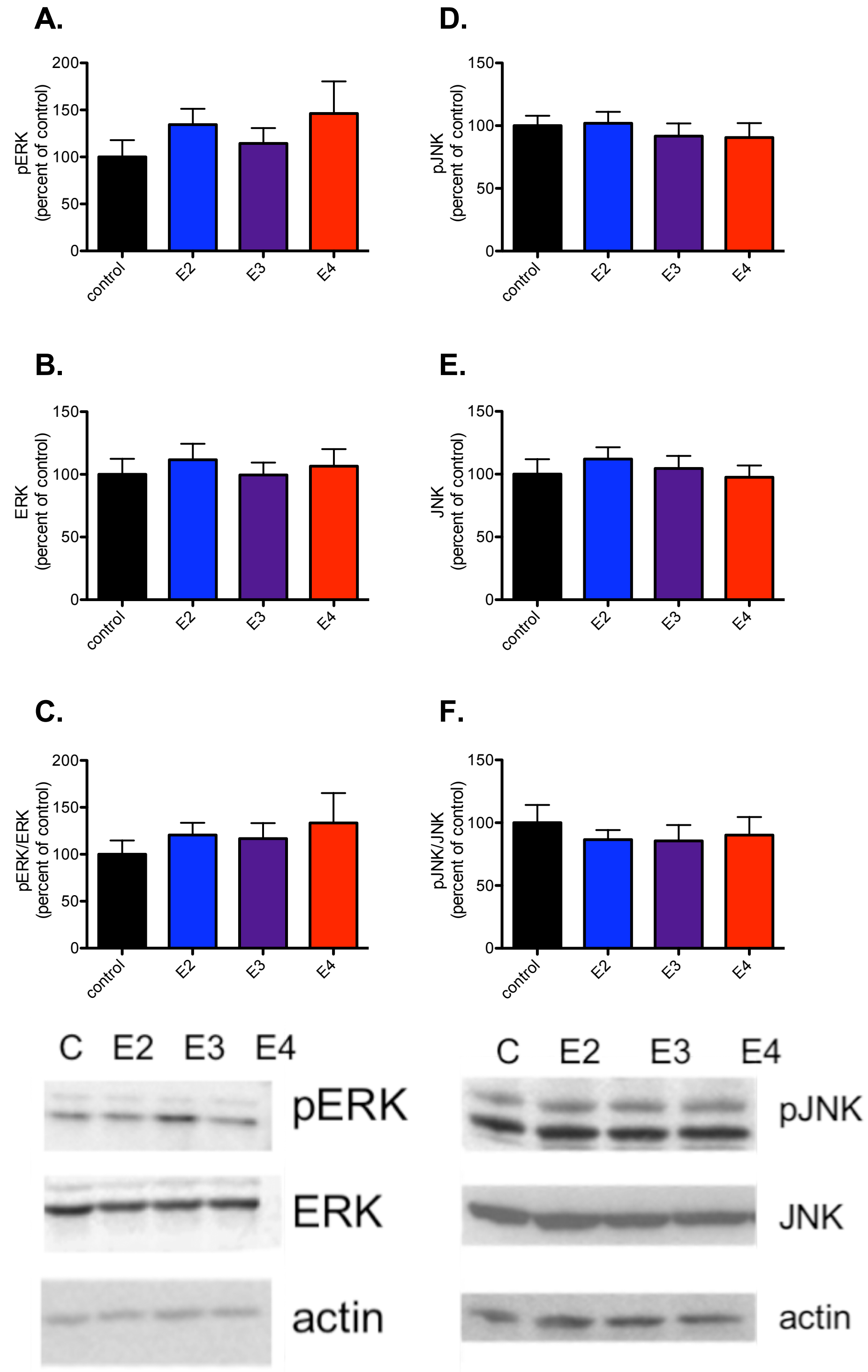


Figure 8: Effects of acute apoE isoform exposure on ERK1/2 and JNK1/2 activation
CA1 of apoE-deficient animals treated with 100 nM rhapoE isoforms (n=6 for each).
Quantification of A) pERK, B) ERK, and C) pERK/ERK ratio
Quantification of D) pJNK, E) JNK, and F) pJNK/JNK ratio.
Representative western blots showing levels of pERK, ERK, pJNK, JNK, and actin. control= C, black, rhapoE2 = E2, blue, rhapoE3 = E3, purple, rhapoE4 = E4, red. Data expressed as mean ± SEM.

Discussion

The identification of apoE as an isoform specific risk factor for AD in 1993 brought much excitement and speculation to the field (Corder, et al., 1993; Rebeck, et al., 1993). Due to the localization of apoE and its receptors within senile plaques, many hypothesized that apoE isoforms selectively altered the clearance of Aβ or the formation of plaques (Arelin, et al., 2002; Carter, 2005; Jiang, et al., 2008; Koistinaho, et al., 2004). However, there are also numerous studies that implicate proper apoE function in other neuronal functions such as calcium homeostasis (Z. Qiu, et al., 2004), neurite outgrowth (Ji, et al., 2003; Nathan, et al., 1994; Nathan, et al., 2002), neurotransmission (Kitamura, et al., 2004; Krugers, et al., 1997; Krzywkowski, et al., 1999), and signal transduction (Hoe, et al., 2005; Hoe & Rebeck, 2005). Changes in signal transduction based on apoE isoform are particularly intriguing to AD researchers as these differences may contribute to alterations seen in AD risk.

While changes in AD risk may be due to chronic exposure to apoE isoform, there is a precedent for the study of acute apoE isoform signaling. ApoE expression levels increase after percussive brain injury in rats versus sham treated animals (Iwata, et al., 2005). Additionally, the presence of apoE4 increases the amount of damage seen in the brain following ischemic injury in mice (Mori, et al., 2003). Administration of an apoE peptide composed of amino acids 133-149 during closed head injury improves functional outcome as well as reduces microglial activation and brain inflammation and suppresses Aβ42 expression (H. Wang, et al., 2007). Isolation of apoE signaling events by the use of

acute isoform exposure will add tremendously to our knowledge of apoE signaling and complement previous studies.

In this study, I used hippocampal slices from apoE-deficient animals as a medium for testing the effects of recombinant human apoE isoforms on synaptic plasticity and signal transduction. This allowed for the isolation of the effect of apoE isoforms without complications due to the presence of murine apoE. Previous studies of apoE-deficient animals have shown minimal, if any, alterations in synaptic function. ApoE-deficient animals develop normally, but can show decreased synaptic density with age (Masliah, et al., 1995). Krywkowski *et al.* (1999) demonstrated a defect in CA1 LTP in apoE-deficient animals with one train of TBS, but this effect was not seen with 3 trains of TBS. Likewise, I was able to induce robust potentiation in apoE-deficient slices with 5 trains of TBS. However, in perforant path apoE-deficient animals show reduced LTP elicited by 3 trains of 100 Hz stimulation in comparison to murine- and apoE3-expressing animals (Trommer, et al., 2004). This suggests that apoE may have differential effects based on hippocampal area; further study will be required to elucidate the mechanism of these differences.

While apoE-deficiency may result in some changes in synaptic plasticity, this study was focused on the additional effects that the presence of apoE isoform would cause. There were no gross defects in brain anatomy observed in apoE-deficient animals. Slices obtained from apoE-deficient animals displayed robust LTP induction with no changes in short-term plasticity or synaptic transmission. This is beneficial to this study

as it allows for us to study a risk factor for AD without the confounding neuro-degeneration that plagues other mouse models of AD.

Addition of rhapoE2, rhapoE3, or rhapoE4 did not alter synaptic transmission or short-term plasticity. While not significantly different from control, LTP induction was increased with application of rhapoE4 and decreased with application of rhapoE2. There was a significant difference in LTP induction between rhapoE2 and rhapoE4. ApoE3 application did not significantly alter LTP induction from control levels. Thus acute application of apoE has an isoform-dependent effect on the induction of LTP. This suggest a role for apoE as an isoform-specific signaling ligand that modulates the machinery responsible for synaptic plasticity.

Acute stimulation of the LDL receptor family by ligands like reelin can activate signal transduction pathways that modulate synaptic plasticity. This modulation is accomplished by activation of specific signaling pathways that alter the responsiveness of the postsynaptic neuron. Reelin binding to apoER2 and VLDLR specifically induces phosphorylation of NMDA receptor subunits via SFK activation resulting in increased NMDA receptor conductance and enhanced LTP (S. Qiu, Zhao, et al., 2006). As ligands for these same receptors, apoE is hypothesized to be another signaling molecule that can modulate synaptic plasticity. By acting through apoER2, apoE could share the capacity to alter NMDAR function. However, unlike reelin, application of exogenous apoE isoforms did not increase NMDAR field potentials. Both rhapoE2 and rhapoE4 decrease NMDAR field potentials, a finding that contrasts their dissimilar effects on LTP induction. In light of the physical (Beffert, et al., 2005; Hoe, Pocivavsek, Chakraborty, et

al., 2006) and signaling (Hoe, et al., 2005; S. Qiu, Zhao, et al., 2006; Sinagra, et al., 2005) link between lipoprotein receptors and NMDA receptors, apoE application may have isoform-specific effects on either the surface expression or function of NMDA receptors that results in a decrease in NMDAR field potentials

The disparity between an increase in LTP coupled with a decrease in NMDAR field potentials in the presence of rhapoE4 could indicate apoE-isoform specific changes in other signal transduction pathways central to LTP. Likewise, the decreases in NMDAR field potentials in the presence of rhapoE2 and rhapoE4 but not rhapoE3 may indicate changes in NMDAR function, such as that seen with alterations in tyrosine phosphorylation. Acute exposure to exogenous reelin increases NMDAR tyrosine phosphorylation (S. Qiu, Zhao, et al., 2006). However, acute exposure to rhapoE isoforms does not have the same effect (figure 7). There are no significant differences in pNR2A/NR2A or pNR2B/NR2B ratios between control, rhapoE2, rhapoE3 or rhapoE4 treated slices.

Although there were no significant differences in NMDAR subunit tyrosine phosphorylation, there was a trend towards a decreased ratio in the presence of apoE isoforms. Importantly, this study looked at total tyrosine phosphorylation, not specific sites. Further study will be required to determine if apoE isoform alters phosphorylation of specific residues, and how this altered phosphorylation correlates with alterations in NMDAR mediated field potentials and LTP induction. In addition, while this trend towards decreased phosphorylation may underlie the changes in NMDAR field potentials seen in the presence of rhapoE2 and rhapoE4, the same change in phosphorylation was

observed in the presence of rhapoE3 which did not alter NMDAR field potentials. This indicates that other factors may be altering NMDAR function, such as changes in receptor localization or a reduction in the number of silent synapses that ultimately change LTP induction but are not measurable by the assays performed in this study.

In addition to changes in NMDAR function, apoE isoforms have the potential to significantly alter signal transduction pathways that are central to LTP induction. Two of the more obvious candidates are the ERK1/2 and JNK1/2 pathways. Previous investigations have revealed that recombinant apoE and apoE-peptide can alter activation of ERK1/2 and JNK1/2 in neuronal cultures (Hoe, et al., 2005). These actions are also tied to NMDA receptor function, which stems from the intimate association of certain LDLRs with NMDA receptor subunits (Hoe, Pocivavsek, Chakraborty, et al., 2006). To connect these changes in signal transduction with alterations in LTP, I probed for changes in ERK1/2 and JNK1/2 activation in apoE-deficient CA1 samples that had been treated with rhapoE isoforms. Unlike the previous study, I did not see any changes in these signal transduction pathways with apoE isoform exposure. This may reflect basal differences in the treatment of apoE and its receptors in neuronal cortical culture and acutely prepared CA1 slices, or may instead stem from more basic experimental differences such as the commercial source of recombinant apoE. Further investigation will be required to distinguish between these possibilities.

Without changes in ERK1/2 and JNK1/2 activation or NMDAR-subunit tyrosine phosphorylation, how can apoE isoform exposure have such dramatic effects on LTP induction? The answer may lie at the junction between the direct effects of apoE

isoforms and the indirect effects of apoE on reelin signaling. ApoE may be simultaneously inducing changes in neuronal function while also altering the ability of reelin to signal via lipoprotein receptors. A recent unpublished study has shown that apoE isoform differentially alters receptor turnover, with apoE4 delaying apoER2 retroendocytosis, or the re-expression of this receptor on the cell surface as an alternative to degradation in the lysosomes (J. Herz, personal communication). This could have a profound impact on NMDAR function, either through the direct physical interaction with apoER2 or though alterations in signal transduction, as illustrated in figure 9. The reduction in surface apoER2 in the presence of rhapoE4 also effectively alters reelin signaling by increasing the ratio of reelin to available receptors. Upon stimulation, slices treated with rhapoE4 have been "primed" due to increased reelin signaling, resulting in a selective increase in LTP induction. This selective difference between rhapoE4 and rhapoE2 could be further exaggerated by apoE-dependent alterations in reelin expression or processing, but this mechanism remains to be tested.

The experimental design of this study imitates previous studies of lipoprotein receptor signaling pathways. The acute exogenous application of rhapoE isoforms is a convenient way to study the signaling capabilities of apoE within a narrow window. This design, however, is not the best approximation of how apoE performs *in vivo*. Many of the results presented in this study may be influenced by the experimental set-up, namely the application of a bolus of non-lipidated apoE in a short period of time. A complementary study of the effects of chronic apoE expression would give further insight into isoform-specific changes to synaptic plasticity.

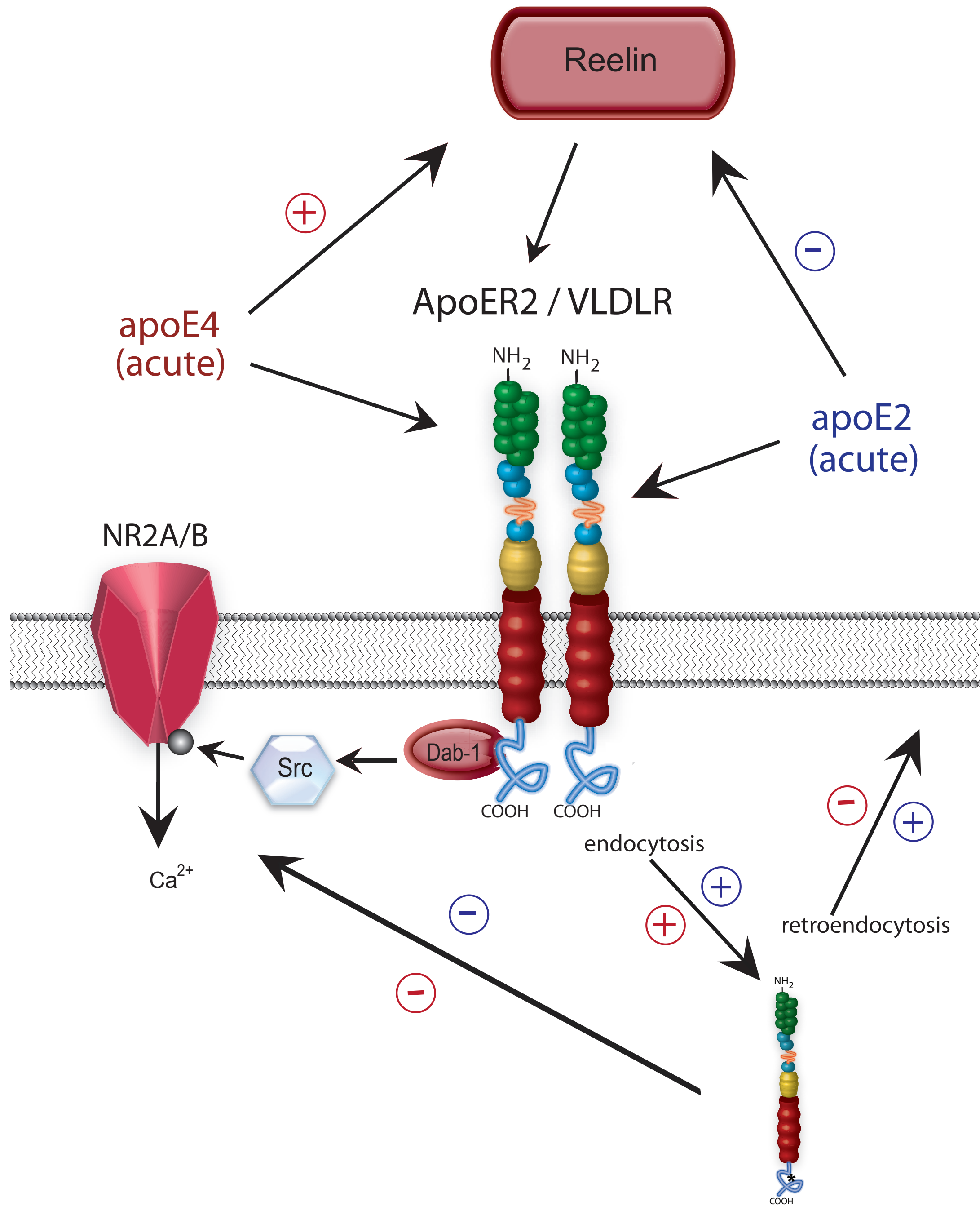


Figure 9: Proposed model of acute apoE isoform effects on synaptic plasticity
Application of apoE2 or apoE4 initiates endocytosis of lipoprotein receptors, including apoER2 and VLDLR. This endocytosis may underly the observed decreases in NMDAR-mediated field potentials, either directly or indirectly. ApoE4 delays retroendocytosis of receptors. In addition, apoE isoform may modulate reelin metabolism, with apoE4 increasing the availability of active reelin fragments. Together these changes can modulate reelin-mediated signaling and thereby alter LTP induction.

CHAPTER III

ISOFORM-DEPENDENT CHANGES IN HIPPOCAMPUS SYNAPTIC FUNCTION WITH CHRONIC APOE ISOFORM EXPRESSION

Introduction

Both acute and chronic manipulations to the signal transduction pathways that center around the LDLR family can alter synaptic plasticity and learning and memory function. As previously mentioned in Chapter II, acute activation of LDLRs can increase LTP while blocking the same receptors inhibits LTP induction (Battey, et al., 1994; Strasser, et al., 2004; Weeber, et al., 2002). In vivo manipulations to this system also result in alterations to LTP. For example, a chronic decrease in reelin levels as seen in the heterozygote reeler mouse (HRM) alters LTP, long-term depression (LTD), and associative learning (S. Qiu, Korwek, Pratt-Davis, et al., 2006). Likewise, deletion of VLDLR or apoER2 also alters LTP and associative learning performance (Weeber, et al., 2002).

Chronic apoE isoform expression may also alter synaptic plasticity and learning and memory by modulating lipoprotein receptor signaling. An *in vivo* system featuring apoE isoform expression would allow for investigation into these effects. Such a system would also facilitate study of the interactions between LDLRs, apoE, and other ligands such as reelin. Fortunately, a promising system has already been developed: the apoE targeted replacement mouse.

Targeted replacement apoE isoform expressing mice (apoE TR) mice were developed by targeting human apoE exons 2-4 to the complementary region in the mouse genome. This strategy resulted in a chimeric gene possessing human coding sequences in place of mouse without disturbing the known normal murine regulatory sequences (Sullivan, et al., 1997). Initial studies showed that total levels of human apoE3 mRNA was equivalent to levels of murine apoE in wild-type littermates (Sullivan, et al., 1997). This equal expression as well as the high degree of conservation between murine and human apoE receptors allows for these mice to be used as a model for direct comparison of apoE isoforms (Herz & Bock, 2002).

Subsequent studies, however, indicated that there may be variation in apoE protein levels based on isoform. Within a particular brain region, apoE levels are similar between isoform (Sullivan, et al., 2004). However, this finding appears to be dependent on the methods used to extract and/or quantify apoE levels. ApoE4 levels are equivalent to apoE3 when measured by quantitative ELISA (Sullivan, et al., 2004). When measured by western blot, apoE4 levels are significantly reduced in both apoE TR hippocampal tissue (Ramaswamy, et al., 2005; Riddell, et al., 2008) and in conditioned media from human astrocytomas (Riddell, et al., 2008). The significance of these findings remain open to interpretation. However, these results do introduce the possibility that there may be isoform-specific variation in release or reuptake of apoE in these animals. This possibility is just now being explored; preliminary evidence indicates that apoE4 inhibits retroendocytosis of apoER2, thus altering receptor expression patterns and apoE levels (J. Herz, personal communication).

Despite the obvious involvement of lipoprotein receptors and their ligands in synaptic plasticity, the characterization of hippocampus synaptic plasticity in the apoE TR has only begun in the past few years. The most complete past investigation focused on perforant path LTP. In this study, LTP induction varied by isoform: wild-type = apoE3 TR > apoE-deficient = apoE2 TR > apoE4 TR (Trommer, et al., 2004). The effects of apoE on LTP appear to depend on the hippocampal region that is being investigated. In a similar, independently developed line of apoE TR animals, young apoE4 TR mice have an enhancement of CA1 LTP over wild-type controls (Kitamura, et al., 2004). Additional comparison of the effect of chronic apoE isoform expression on CA1 synaptic plasticity in the remaining apoE TR lines was unfortunately absent.

In this study, I investigated the effects of chronic apoE isoform expression on CA1 synaptic plasticity in the adult mouse. I used field recording techniques to monitor changes in synaptic plasticity between apoE2, apoE3, and apoE4 TR mice as well as between apoE-deficient and murine apoE-expressing animals. Together with these electrophysiological techniques, I also observed biochemical changes due to apoE isoform expression. These findings allowed me to determine a preliminary mechanism by which chronic apoE isoform expression alters hippocampus synaptic plasticity.

Methods

Animal maintenance

ApoE2, apoE3 and apoE4 targeted replacement animals were obtained from a colony maintained at Taconic (Hudson, NY). ApoE knock-out and C57BL/6J (wild-type) animals were obtained from Jackson Laboratories (Bar Harbor, ME). All animals were fully backcrossed on a C57BL/6J background. Animals were housed in a standard 12 hour light cycle. Animals were bred for experimentation in a homozygous fashion and maintained in accordance with the Vanderbilt University Institutional Animal Care and Use Committee protocol. Animals were given *ad libitum* access to standard mouse chow (LabDiet, PMI Nutrition International) and water.

Electrophysiology

Hippocampus slices were prepared from 3- to 5-month old mice for field electrophysiology. The animals were sacrificed by cervical dislocation and the brain was rapidly removed and placed in ice-cold high sucrose cutting saline solution containing (in mM) 110 sucrose, 60 NaCl, 3 KCl, 28 $NaHCO_3$, 1.25 NaH_2PO_4, 5 glucose, 0.6 ascorbate, 7 $MgCl_2$, and 0.5 $CaCl_2$ with continuous perfusion of 95% O2/5% CO2 Horizontal 400μm sections were cut in high sucrose cutting solution using a vibratome. Slices were maintained in cold, oxygenated cutting solution until dissection of the hippocampus from the surrounding tissue.

After dissection, the hippocampus slices were transferred to room temperature cutting solution diluted 1:1 with artificial cerebral spinal fluid (ACSF). ACSF contains (in mM) 125 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 25 glucose, 1 $MgCl_2$, and 2 $CaCl_2$. Slices were maintained in this solution with constant 95% O_2/5% CO_2 perfusion for 10 min before transferring to the interface brain slice recording chamber (Fine Science Tools, San Francisco, CA).

The recording chamber was maintained at 30°±0.5°C with a laminar ACSF flow rate of approximately 1 mL/min. Field excitatory postsynaptic potentials (fEPSPs) were recorded from area CA1 *stratum radiatum* via glass micropipettes pulled to an approximate 1 μm tip diameter (1-4 MΩ) and filled with ACSF. Responses were generated by stimulation of fibers arising from the CA3 region. Stimulating electrodes consisting of formvar-coated nichrome wire delivered biphasic stimulus pulses (1-15 V, 100 μsec duration, 0.05 Hz). Delivery of stimulation, controlled by pClamp 9.0 software (Axon Instruments, Forster City, CA), was via the Digidata 1322A interface (Axon Instruments) and a stimulus isolator (model 2200, A-M Systems, Sequim, WA). Signals were amplified using a differential amplifier (model 1800, A-M Systems), filtered at 1 kHz and digitized at 10 kHz. For all experiments, baseline stimulus intensity was set at the level that elicited 40-50% of the maximum fEPSP response as determined from the input-output curve.

Paired-pulse facilitation was induced by delivery of two stimuli in close temporal proximity. Intervals between stimuli began at 20 ms and increased to 300 ms in 20 ms

intervals. Paired-pulse facilitation was calculated by the percent facilitation of fEPSP slope of second response from first response.

Long-term potentiation (LTP) was induced by theta-burst protocol. Theta-burst LTP protocol consisted of five trains of 10 bursts at a 5Hz frequency with each burst consisting of 4 stimulations delivered at 100Hz and an inter-train interval of 20 seconds. NMDAR-independent LTP was induced by 2 one-second trains of stimulation delivered at 200Hz; concurrent application of 100 μM 2-amino-5-phosphonovaleric acid (APV) confirmed NMDAR independence.

Electrophysiological data was also analyzed using one-way ANOVA with Bonferroni's post hoc tests. Significance was set at $p<0.05$ for all tests.

Biochemistry

Slices were obtained from apoE2 TR, apoE3 TR, apoE4 TR, apoE-deficient, and C57BL/6J mice in an identical fashion as for electrophysiology (see above). The slices were frozen on dry ice and CA1 dissected unless otherwise stated.

ApoE/ApoER2 levels - Bilateral dissections of the whole hippocampus from aged (1 year old) animals were performed. Brain tissue was rapidly dissected and flash frozen on dry ice. Tissue was homogenized in NP-40 lysis buffer containing (in mM) 50 Tris-HCL ph 8.0, 150 NaCl, 1 EDTA, 1 PMSF, 1 Na_3VO_4, 1 NaF, 1 μg/mL each of aprotinin, leupeptin and pepstatin, and 1% NP-40. Protein concentration was determined by Bradford Assay (BioRad). For western blot analysis, 10 μg of protein was resolved by SDS-PAGE on 4-20% gradient Tris-HCl gels (BioRad). The proteins were then

transferred to PVDF membranes. Membranes were probed with goat anti-human apoE (Academy Bio-medical, Houston TX), rabbit anti-apoER2 (a gift of Drs. Gary Olson and Ray Burk, Vanderbilt University), and rabbit anti-actin (Sigma) diluted in 0.24% I-block (Tropix). Membranes were developed using HRP-conjugated secondary antibodies and enhanced chemiluminescence.

NMDAR subunit phosphorylation - Pooled CA1 tissue was sonicated in modified RIPA buffer (Tris/HCl pH 7.4, 2 mM EDTA, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% triton X100, 1X phosphatase inhibitors I and II (Sigma), and 1X complete protease inhibitors (Sigma). Protein concentrations were determined by BCA protein assay (BioRad). For immunoprecipitation, a total of 400 μg of protein lysate was used to immunoprecipitate either NMDA-NR2A (NMDAε1, Santa Cruz Biotechnology) or NMDA-NR2B (NMDAε2, Santa Cruz Biotechnology) overnight at 4°C with agitation. Protein A/G magnetic beads (New England BioLabs) were added to each reaction (25 ul bead slurry/reaction) and samples were incubated for 2 hours at 4°C with agitation. Following three wash cycles, the protein was eluted with 1X Laemmli sample buffer separated by SDS-PAGE on 4-15% Tris-HCl gradient gels (BioRad) and transferred to PVDF membranes. Membranes were probed with goat anti-NMDAε1, rabbit anti-NMDA, or mouse anti-pTyr (PY99) (Santa Cruz Biotechnology) in 2% BSA-TBST. Membranes were developed using HRP-conjugated secondary antibodies and enhanced chemiluminescence. For western blot analysis, ten μg of protein was resolved by SDS-PAGE on 4-15% Tris-HCL gradient gels (BioRad) and transferred to PVDF membrane. Membranes were probed with rabbit anti-NR2A, rabbit anti-NR2B (Upstate), mouse anti-

phosphotyrosine, clone 4G10 and mouse anti-NR1 (Millipore) diluted in 0.24% I-block (Tropix). Membranes were developed using HRP-conjugated secondary antibodies and enhanced chemiluminescence.

ERK/JNK activation – Pooled CA1 tissue was homogenized in NP-40 lysis buffer containing (in mM) 50 Tris-HCl pH 8.0, 150 NaCl, 1 EDTA, 1 PMSF, 1 Na_3VO_4, 1 NaF, 1 µg/mL each of aprotinin, leupeptin and pepstatin, and 1% NP-40. Protein concentration was determined by Bradford Assay (BioRad). Ten µg of protein was resolved by SDS-PAGE on 10% Tris-HCl gels. Membranes were probed with rabbit anti-ERK1/2, anti-ERK1/2 pTpY$^{185/187}$, anti-JNK1/2, anti-JNK1/2 pTpY$^{183/185}$ (Invitrogen) diluted in 0.24% I-block. Membranes were developed using HRP-conjugated secondary antibodies and enhanced chemiluminescence.

Optical density of immunoreactivity was quantified by densitometry using Image J software (NIH).

Results

LTP induction in apoE TR animals is isoform dependent

Much like apoE-deficient animals, apoE TR mice develop normally without gross pathological changes to brain organization. Hippocampal slices from 3-5 month old animals appear equivalent to wild-type hippocampi on a gross level. This suggests that isoform variation in apoE signaling does not significantly impact neuronal migration during development. This stands in contrast to reelin-deficient mice (D'Arcangelo, et al.,

1999), giving the apoE TR and apoE-deficient mice a clear advantage when examining synaptic function.

Supporting the lack of structural changes, hippocampus area CA1 synaptic transmission does not vary significantly with apoE isoform expression (figure 10A). These data ensure an important parameter in plasticity studies: that specific input elicits an equivalent output regardless of genotype. Thus, subsequent variation in plasticity can be attributed to apoE isoform rather than changes in CA1 synaptic connectivity. Determination of short-term plasticity using paired-pulse facilitation revealed typical percent facilitation between apoE TR, apoE-deficient and wild-type mice (figure 10B).

Using five trains of theta burst stimulation, I successfully induced potentiation in wild-type (C57BL/6J), apoE-deficient, apoE2, apoE3, and apoE4 TR mice. The magnitude of potentiation, however, was dramatically different between apoE isoforms. Immediately following stimulation and persisting for the duration of the experiment, apoE4 TR mice had increased LTP induction (figure 10C). Potentiation in apoE4 TR mice was significantly increased from that seen in the apoE2 TR (figure 10D; ANOVA, p=0.0198). LTP induction in the apoE-deficient mice was intermediate between apoE2 TR and apoE4 TR, but this trend was not significant. There was no significant difference in the magnitude of LTP induction between wild-type, apoE-deficient, and apoE3 TR animals. Interestingly, the finding of increased LTP in the apoE4 TR is contradictory to perforant path LTP in the same animals (Trommer, et al., 2004), suggesting a differential role for apoE in perforant path and CA1 synaptic plasticity.

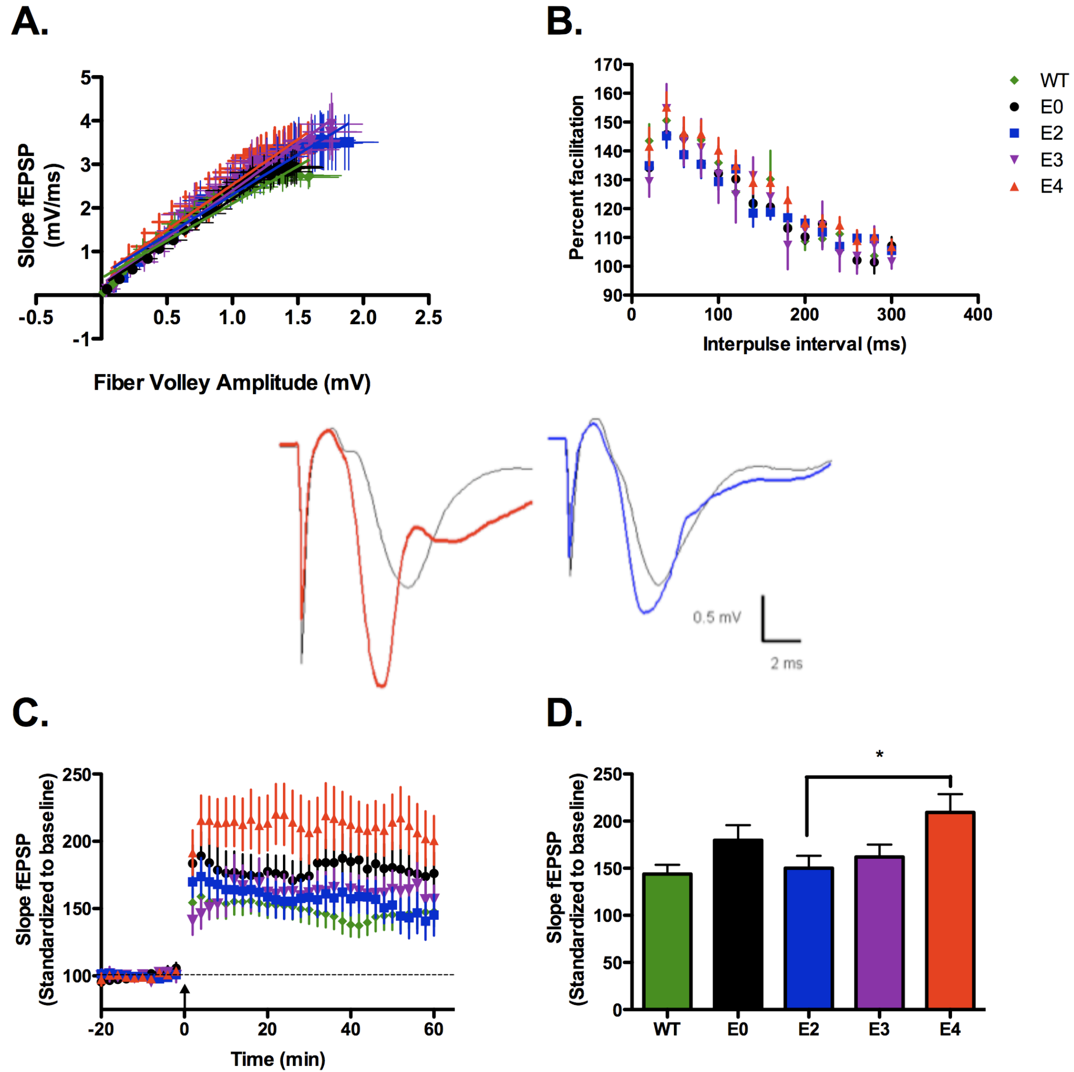


Figure 10: ApoE TR animals show altered LTP induction without changes in synaptic transmission.

A) Input-output curve generated from the slope fEPSP versus fiber volley amplitude measured at increasing stimulus intensities. B) Paired pulse facilitation. Second stimuli delivered at 20 ms intervals from 20 to 300 ms from first stimuli. Percent facilitation of fEPSP slope of second response as percentage of first response. C) Long-term potentiation induced by 5 trains of theta-burst stimulation (arrow). Expressed as slope of fEPSP, standardized to the first 20 minutes of recording. Representative traces 5 minutes before (black lines) and 40 minutes after (colored lines) stimulation for apoE4 (red) and apoE2 TR (blue). Scale: 0.5 mV, 2 ms. D) Average potentiation of last 20 minutes of recording. C57BL/6J = WT, green, n=12; apoE-deficient = E0, black, n=12; apoE2 TR = E2, blue, n=17; apoE3 TR = E3, purple, n=6; apoE4 TR = E4, red, n=14. Data expressed as mean ± SEM. *$p<0.05$, ANOVA with Bonferroni's posttest.

Previous studies have shown that alterations in apoER2 expression (Weeber, et al., 2002) and reelin expression (S. Qiu, Korwek, Pratt-Davis, et al., 2006) can adversely affect spatial memory as well as LTP induction. To ensure that changes in receptor availability or ligand concentration were not underlying the isoform-specific effects on LTP in the apoE TR mouse, I probed for changes in overall expression levels of apoER2, the main apoE receptor in the brain. Isolated hippocampal tissue was probed for apoER2 expression using western blot analysis. Figure 11 indicates that apoE2, apoE3 and apoE4 TR animals exhibit no alterations in apoER2 levels in the hippocampus (ANOVA, p=0.8269). ApoE-deficient and murine apoE-expressing animals are also equivalent in receptor expression (t-test, p=0.6175). In addition, I also verified that targeted replacement did not alter overall apoE expression levels in the hippocampus as there were no significant differences in hippocampal apoE levels between apoE2, apoE3, and apoE4 TR mice (figure 11, ANOVA, p=0.6912).

Effect of apoE isoform expression on NMDA receptors

Theta-burst induced LTP in the hippocampus requires proper NMDAR function (Larson & Lynch, 1988; Larson, et al., 1986). Signal transduction via apoE receptors can alter this function by influencing NMDA receptor maturation (S. Qiu & Weeber, 2007; Sinagra, et al., 2005), increasing NMDAR currents (S. Qiu, Zhao, et al., 2006) and activating other signaling pathways that involve NMDA receptor function (Beffert, et al., 2005; Hoe, Pocivavsek, Chakraborty, et al., 2006; S. Qiu, Zhao, et al., 2006). The association between NMDAR function and apoE receptors lead to the hypothesis that the

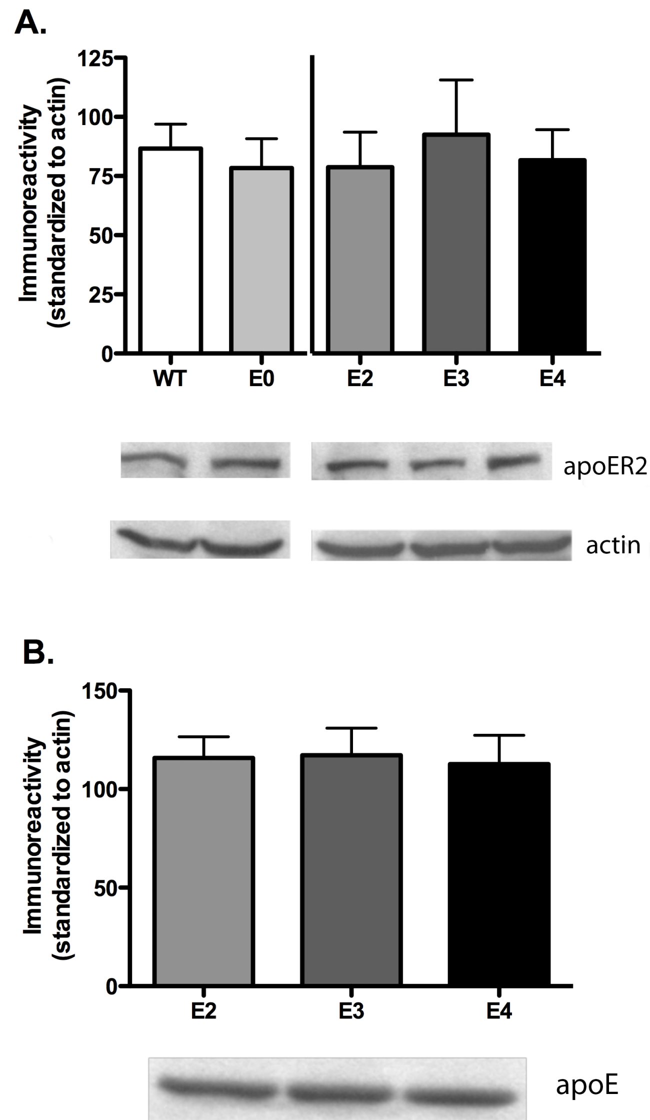


Figure 11: ApoE isoform expression does not affect apoER2 expression levels
Representative western blots showing levels of apoE (A) and apoER2 (B) immunoreactivity in whole hippocampus of aged animals. Quantification of immunoreactivity standardized to actin (n=8 for WT, E0, E2, E4, n=5 for E3). C57BL/6J (WT, white), apoE-deficient (E0, light grey), apoE2 TR (E2, medium grey), apoE3 TR (E3, dark grey), apoE4 TR (E4, black). Data expressed as mean ± SEM.

observed alterations in LTP may be due to apoE isoform-specific changes in NMDA receptor function.

To test this hypothesis, NMDA receptor-independent LTP was induced by delivering two one-second trains of 200 Hz stimulation concurrent with application of the NMDA receptor antagonist APV (100 μM). This induced long-lasting potentiation in wild type, apoE-deficient, and apoE TR animals. The isoform-dependent alterations in LTP induction, however, were eliminated with this protocol (figure 12), suggesting that apoE isoform expression may be modifying NMDAR function.

As apoE4 increased LTP in a NMDAR-dependent manner similar to what is seen with reelin application, I hypothesized that apoE4 expression may also be increasing tyrosine phosphorylation of NMDA receptor subunits. Reelin application significantly increases both NR2A and NR2B tyrosine phosphorylation in a Src-dependent manner (S. Qiu, Zhao, et al., 2006). To test hypothesis that apoE isoform may be altering the state of NMDAR subunit tyrosine phosphorylation, I attempted to immunoprecipitate the NMDA receptor subunits NR2A and NR2B from area CA1 of the hippocampus of apoE TR, wild-type, and apoE-deficient animals then probe for tyrosine phosphorylation.

When immunoprecipitating NR2A, the levels of detected NR2A were significantly reduced in apoE4 TR animals (figure 13B, ANOVA $p=0.0050$). This was accompanied by a significant increase in pNR2A as measured by pTyr immunoreactivity in the apoE4 TR versus apoE2 and apoE3 TR (figure 13A, ANOVA $p=0.0023$). The resulting ratio of tyrosine phosphorylated NR2A to total NR2A was significantly increased in apoE4 TR over apoE2 TR (figure 13C, ANOVA $p=0.0406$). In contrast, there

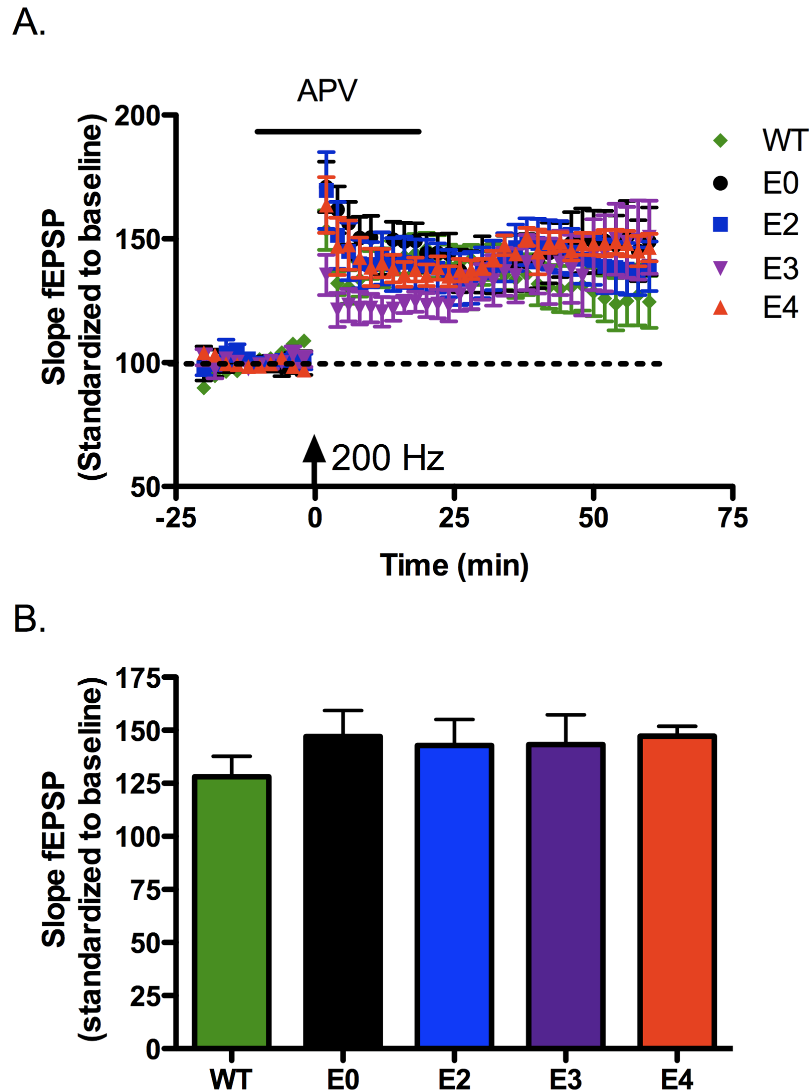


Figure 12: NMDA receptor-independent LTP is not affected by apoE isoform expression

A. Long term potentiation induced by 2 trains of 200 Hz stimulation (arrow) with application of 100 µM APV for 5 minutes before and 20 minutes after 200 Hz stimulation. Expressed as slope of fEPSP, standardized to the first 20 minutes of recording. B. Average potentiation of last 20 minutes of recording. C57BL/6J = WT, green, n=5; apoE-deficient = E0, black, n=8; apoE2 TR = E2, blue, n=8; apoE3 TR = E3, purple, n=7; apoE4 TR = E4, red, n=6. Data expressed as mean ± SEM.

were no significant differences in pNR2A or NR2A between wild-type and apoE-deficient animals.

Detected pNR2B levels also were significantly higher in apoE4 TR versus apoE2 and apoE3 TR animals (figure 13D, ANOVA p=0.0004). Combined with a reduction in NR2B levels in the apoE4 TR (figure 13E, ANOVA p=0.0282), this resulted in a significant increase in the ratio of tyrosine phosphorylated NR2B to total NR2B in the apoE4 TR (figure 13F, ANVOA p=0.0029). As the magnitude of this increase was very large, approximately 400% of levels seen in apoE-deficient animals, it was necessary to validate these findings in a parallel study not involving immunoprecipitation.

In order to ensure that excessive phosphorylation in the apoE4 TR samples was not altering the ability to immunoprecipitate NMDAR subunits, I subjected CA1 samples from apoE TR animals to western blot analysis of NR1, NR2A, NR2B, and tyrosine phosphorylation. In these samples, there were no significant differences in the ratio of NR2A to NR1 or NR2B to NR1 between any of the apoE TR animals (figure 14A,B ANOVA NR2A/NR1 p=0.4828, NR2B/NR1 p=0.0941). Total tyrosine phosphorylation at the molecular weight corresponding to NR2A and NR2B was also unchanged between apoE2, apoE3, and apoE4 TR animals (figure 14C, ANOVA p=0.1117).

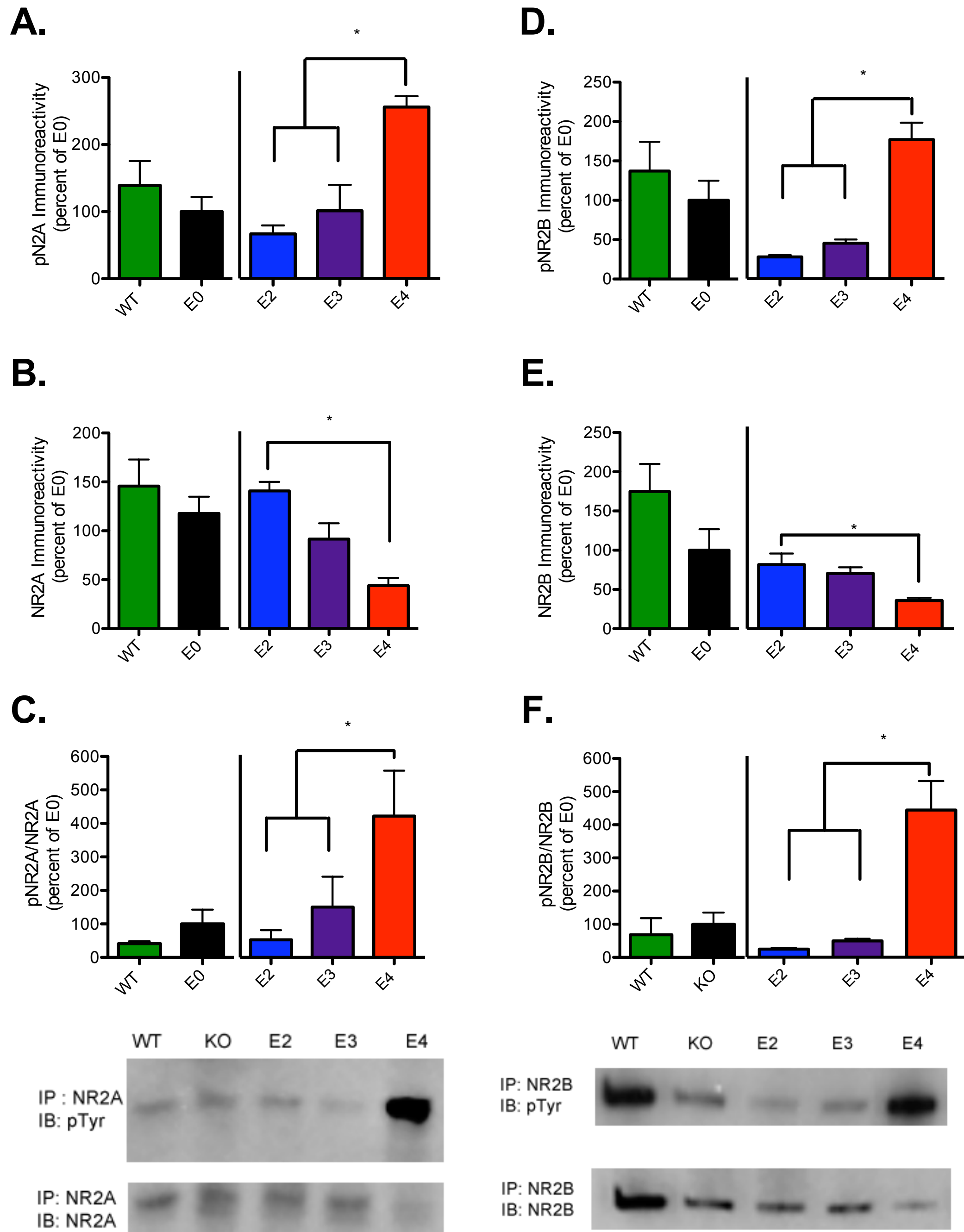


Figure 13: Immunoprecipitation of NMDAR subunits is hindered in apoE TR animals

Immunoprecipitation of NR2A from CA1 followed by immunodetection of A) pNR2A with anti-pTyr antibody and B) NR2A. C) Ratio of pNR2A/NR2A. Immunoprecipitation of NR2B from CA1 followed by immunodetection of D) pNR2B with anti-pTyr antibody and E) NR2B. F) Ratio of pNR2B/NR2B. Representative blots show immunodetection of pTyr and NR2A or NR2B. C57BL/6J = WT, green; apoE-deficient = E0, black; apoE2 TR = E2, blue; apoE3 TR = E3, purple; apoE4 TR = E4, red. Data expressed as mean ± SEM. *$p<0.05$ ANOVA with Bonferroni's posttest.

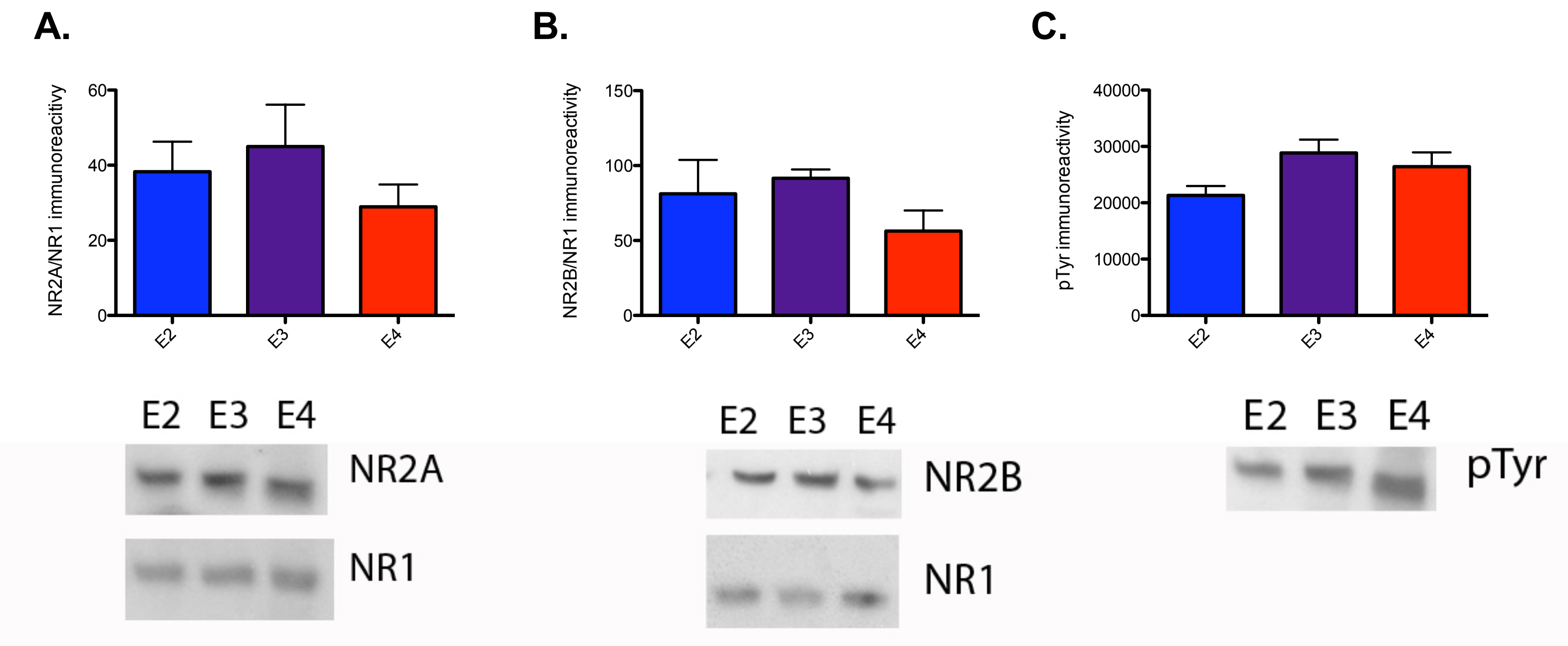


Figure 14: Effect of chronic apoE isoform expression on NR2A, NR2B levels and tyrosine phosphorylation
A. Levels of NR2A immunoreactivity, standardized to NR1 immunoreactivity, from CA1 of apoE TR animals. B. Levels of NR2B immunoreactivity, standardized to NR1 immunoreactivity, from CA1 of apoE TR animals. C. Levels of phosphotyrosine immunoreactivity at the molecular weight corresponding to NR2A and NR2B from CA1 of apoE TR animals. apoE2 TR (E2, blue), apoE3 TR (E3, purple), apoE4 TR (E4, red). Data expressed as mean ± SEM.

ApoE isoform expression alters signal transduction

Previous studies of acute apoE application in neuronal culture have shown that apoE can activate ERK1/2 phosphorylation in an isoform-dependent manner (Hoe, et al., 2005) and it is know that ERK1/2 activation is important for synaptic plasticity and learning and memory. However, we have yet to see any activation of the ERK signaling pathway with reelin application (unpublished observation), or with acute application of rhapoE isoforms. It is likely, however, that prolonged exposure to endogenous apoE isoform can alter activation of signal transduction pathways and thereby influence synaptic plasticity.

The effect of apoE isoform expression on ERK signaling was investigated by isolating CA1 tissue and probing by western blot analysis for pERK1/2 and ERK1/2. While there was no change in pERK between wild-type and apoE-deficient animals, pERK was significantly increased in apoE4 TR over both apoE2 and apoE3 TR animals (figure 15A, ANOVA p=0.0003). There were no corresponding changes in ERK levels in any of the animals (figure 15B, WT vs E0 t-test p=0.4788, E2 vs E3 vs E4 ANOVA p=0.3922). Therefore, the ratio of pERK to ERK was significantly increased in apoE4 TR versus apoE2 TR and apoE3 TR animals and there were no significant differences between apoE-deficient and murine apoE-expressing animals (figure 15C, WT vs E0 t-test p=0.0.0547, E2 vs E3 vs E4 ANOVA p=0.0005).

Activation of JNK was altered by both the absence of apoE and the presence of apoE isoforms. There was significantly less pJNK activation in apoE-deficient animals versus wild-type (figure 15D, t-test p=0.0003). pJNK levels were significantly increased

in apoE4 TR versus apoE2 TR and apoE3 TR animals (figure 15D, ANOVA p=0.0075). There were no significant differences in JNK levels (figure 15E, WT vs E0 t-test p=0.0.9167, E2 vs E3 vs E4 ANOVA p=0.4572). Thus there was a significant difference in pJNK/JNK ratio between wild-type and apoE-deficient animals (figure 15F, t-test p=0.0015). The pJNK/JNK ration in apoE4 TR animals was also significantly different from that seen in apoE2 TR and apoE3 TR animals (ANOVA p=0.0025).

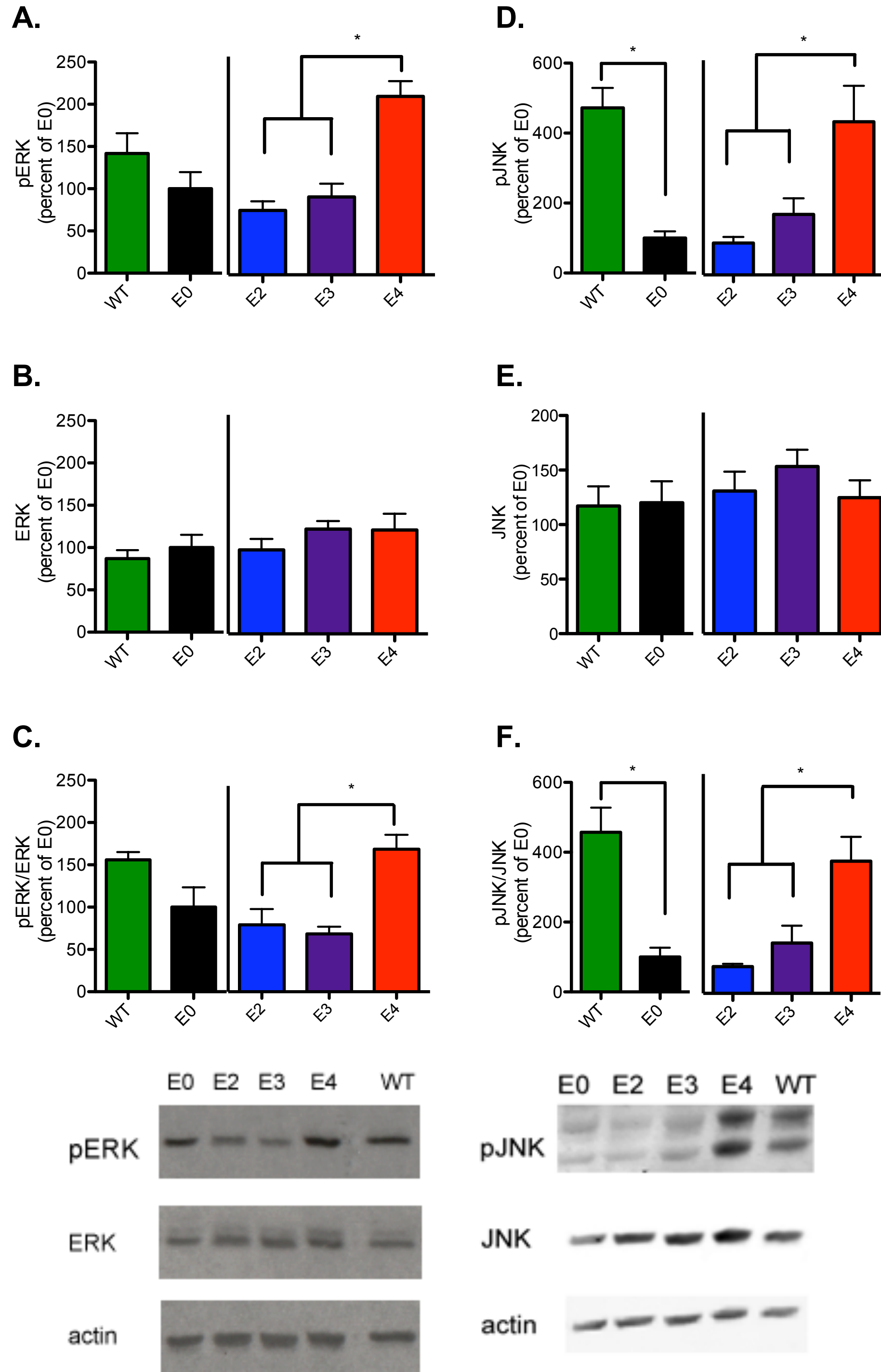


Figure 15: Chronic apoE isoform expression alters activation of ERK1/2 and JNK1/2
Quantification of levels of A) pERK or B) ERK immunoreactivity in CA1 of apoE TR, wild-type, and apoE-deficient animals. C) pERK/ERK ratio.
Quantification of levels of D) pJNK or E) JNK immunoreactivity in CA1 of apoE TR, wild-type, or apoE-deficient animals. F) pJNK/JNK ratio. Quantification of immunoreactivity normalized to background (n=5). C57BL/6J (WT, green), apoE-deficient (E0, black), apoE2 TR (E2, blue), apoE3 TR (E3, purple), apoE4 TR (E4, red). Data expressed as mean ± SEM. *p<0.05, ANOVA with Bonferroni's posttest.

Discussion

Sporadic AD is, in essence, a chronic disease. Barring a catastrophic event like inheritance of an early-onset gene mutation, an individual only develops AD after a lifetime of living with genetic and environmental risk factors. With the identification of some of these risk factors, such as apoE isoform, the question now is: what is apoE doing in the pre-dementia state and how do we slow or halt disease progression?

The study of chronic apoE isoform exposure is facilitated by the use of the apoE TR mouse. Utilizing the endogenous murine promoter helps preserve endogenous expression patterns regardless of isoform. The high degree of conservation in the apoE receptors also aids these studies by isolating the effects of the presence of human apoE isoforms from the absence of murine apoE. While these animals have been studied by many groups, there are still many unanswered questions about the actions of apoE isoforms in the brain. I designed this study with the following goals: 1) to compare all three human apoE isoform-expressing animals to each other and apoE-deficient to murine apoE-expressing animals in order to gain a more complete picture of the effects of chronic apoE isoform expression and 2) to focus on hippocampus area CA1 to isolate region-specific effects of apoE isoforms and allow for the comparison of my results with the established learning and memory literature.

Previous studies of synaptic plasticity in the apoE TR have focused on the perforant path, finding that LTP induction in apoE2 and apoE4 TR as well as apoE-deficient mice was significantly reduced from that seen in wild-type or apoE3 TR animals (Trommer, et al., 2004). In this study of CA1 LTP, however, the greatest amount of LTP

induction is seen in the apoE4 TR. Interestingly, LTP induction in the apoE2 TR is significantly lower than apoE4; apoE3 TR and apoE-deficient have an intermediary level of LTP induction that is not significantly different from either apoE2 or apoE4.

Based on the previous studies of perforant path LTP, these results were surprising. However, on closer examination it appeared that apoE4 may be acting similarly to exogenously applied reelin. A similar enhancement of LTP is seen with bath application of reelin, and this enhancement correlates with both increased tyrosine phosphorylation of NMDA receptors and cell surface insertion of AMPA receptors (S. Qiu, Zhao, et al., 2006). Preliminary evidence suggests that apoE4 expression occludes the enhancement of LTP by reelin (J. Herz, personal communication). In addition, the lack of isoform-dependent changes in LTP with a NMDAR-independent stimulation protocol helps support the hypothesis that chronic apoE isoform expression is signaling via lipoprotein receptors similar to reelin.

Reelin application increases tyrosine phosphorylation of NMDA receptors (S. Qiu, Zhao, et al., 2006). I initially hypothesized that chronic apoE exposure also increases tyrosine phosphorylation of NMDAR subunits, either through direct signaling or indirect enhancement of reelin signaling. When immunoprecipitating NR2A or NR2B from CA1 samples, there was a significant increase in tyrosine phosphorylation and a significant decrease in detected NR2A/B in the apoE4 TR. This raises questions about the specificity of the immunoprecipitation experiments. For instance, could hyperphosphorylation of NR2A/B interfere with antibody specificity? Are the changes in total protein levels limited to the pool of NR2A/B that is available for

immunoprecipitation? The antibodies used for these immunoprecipitation experiments were raised against epitopes that do not overlap with any of the known, major tyrosine phosphorylation sites for NR2A or NR2B (Kennedy & Manzerra, 2001).

To test the possibility that the immunoprecipitation conditions may be preferentially accessing a specific pool of NMDA receptors, I performed a parallel study with whole CA1 extracts. This revealed that NR2A/NR1 and NR2B/NR1 ratios were equivalent between apoE2, apoE3 and apoE4 TR animals. Additionally, there were no changes in the amount of tyrosine phosphorylation at the molecular weight corresponding to NR2A and NR2B between apoE isoforms in these total denatured samples (figure 14). This is in contrast to the isoform-specific changes seen when immunoprecipitating NR2A or NR2B from the same samples. Therefore, the ability to immunoprecipitate NR2A and NR2B may be hindered by differential treatment of NMDA receptors within neurons due to apoE signaling and the immunoprecipitation protocol is not sensitive enough to overcome these differences.

The current study establishes the effect of chronic apoE exposure on ERK1/2 and JNK1/2 signaling. These pathways are well validated as essential to the underlying mechanisms of LTP (Kanterewicz, et al., 2000; Schmitt, et al., 2005; Selcher, et al., 2003; Sweatt, 2001; Winder, et al., 1999), as well as learning and memory (Cestari, et al., 2006; Fischer, et al., 2007; Giovannini, 2006; Satoh, et al., 2007). Modulation of these pathways by either pharmacological or genetic manipulation has dramatic effects on both synaptic plasticity and memory formation. Thus the enhanced LTP seen with apoE4

expression may be due to the ability of apoE to act as an isoform-specific signaling ligand and alter signal transduction in the hippocampus.

While further study will be necessary to determine the mechanism of apoE-isoform specific alterations to LTP induction, the results presented here suggest that specific changes in signal transduction pathways may underlie these differences in synaptic plasticity. Chronic changes in signaling can result in slight alterations to NMDAR subunit composition, localization or function that cumulatively exert a physiologic effect on LTP. As shown by figure 16, alterations in signal transduction via apoER2 have the potential to impact NMDA receptor function. For instance, apoE4 specifically increases ERK1/2 activation. As reelin is unable to induce ERK1/2 activation through apoER2 or VLDLR, this effect may be mediated by other lipoprotein receptors, likely LRP1. There are additional isoform-specific differences in JNK1/2 activation which may stem from the interaction of apoER2 with JIP. These observed alterations in signal transduction cascades complement NMDAR-dependent changes, culminating in apoE isoform-specific changes in synaptic plasticity.

The exact mechanism of apoE isoform-dependent modulation of CA1 synaptic plasticity is still under investigation. Both apoE isoforms and reelin can bind to the same family of lipoprotein receptors. The role of reelin in synaptic function and memory formation is now well established, and specific apoE isoform expression can have a direct effect on reelin signaling and modulation of synaptic function (J Herz, personal communication). Thus, apoE isoforms may function by changing reelin’s ability to modulate NMDA receptors and activate specific signal transduction pathways by as yet

unknown mechanisms. ApoE expression may also induce specific signaling events through lipoprotein receptors that are distinct from other ligands. Furthermore, changes in apoE availability, due to spatial changes or associations with other proteins, may have a profound affect on synaptic function in the different subfields of the hippocampus. Taken together with the lack of LTP deficit in apoE-deficient mice, the results presented here highlight the role of apoE as a modulator of hippocampal synaptic plasticity.

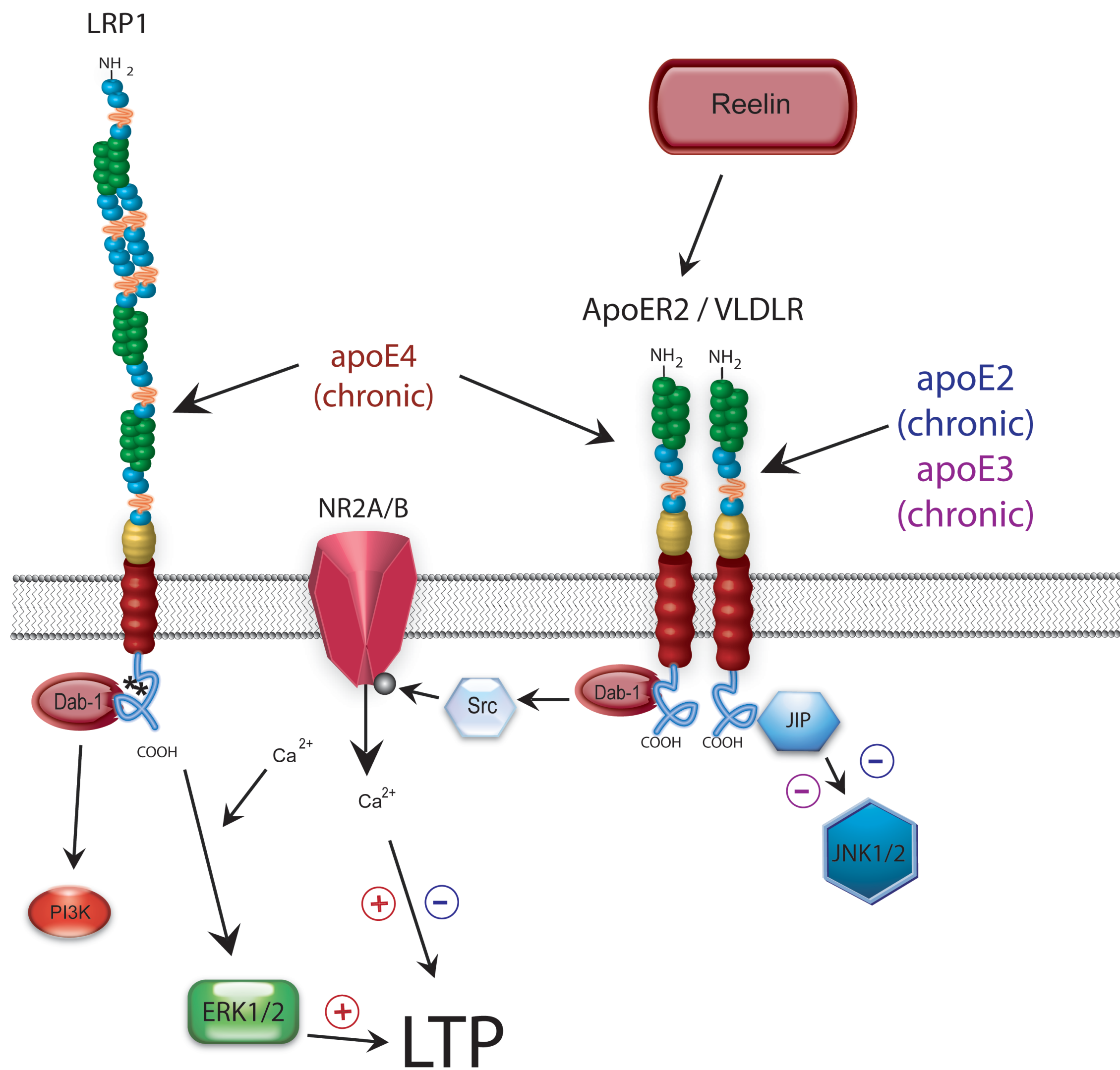


Figure 16: Proposed model of chronic apoE isoform effects on synaptic plasticity
Reelin interacts exclusively with the lipoprotein receptors apoER2 and VLDLR, with a much higher affinity for apoER2. ApoE binds to all lipoprotein receptors and undergoes endocytosis. Chronic apoE4 exposure enhances ERK1/2 activation, likely through interactions with the LRP1 receptor. In contrast, chronic apoE2 and apoE3 expression reduce activation of JNK1/2 and ERK1/2 activation. Together with proper NMDAR function, these changes culminate in alterations in LTP induction with chronic apoE2 isoform expression.

CHAPTER IV

APOE ISOFORM-SPECIFIC EFFECTS ON LEARNING AND MEMORY

Introduction

The synaptic plasticity mechanisms discussed in the previous chapters attempt to decipher the complicated processes of learning and memory. Learning, or acquiring a changed behavior response due to an environmental stimulus, is a highly regulated survival mechanism. Learning is complemented by memory, the storage of learned information, and recall, retrieving that stored information in order to perform the altered behavior (Kandel, et al., 2001). Measuring these processes requires both an understanding of the type of learning and memory being investigated and a prediction of how behavior will change with environmental manipulation.

Learning is typically divided into two main types: unconscious and conscious. Unconscious learning can be recalled consciously, as seen in taste learning and operant conditioning, or unconsciously, such as in habituation, pavlovian conditioning, or motor learning (Sweatt, 2003). Conscious learning is recalled consciously during spatial and declarative learning tasks (Sweatt, 2003). These types of learning and memory tasks require long-term, persistent biochemical changes for maintenance. In contrast, short-term working memory persists in changes in neuronal firing patterns rather than biochemical changes (Kandel, 2001).

This definition of learning corresponds with the classical division of human memory into two categories: declarative (explicit) and non-declarative (implicit) (Kandel, et al., 2001). Non-declarative memory is recalled unconsciously and is typically involved in reflexive training and procedural learning. This involves brain areas such as the neocortex, striatum, amygdala, and cerebellum (Sweatt, 2003). In contrast, declarative memory concerns facts and events and is recalled consciously. These memories require the medial temporal lobe, making them especially vulnerable to temporal lobe damage or neurodegeneration, such as seen in AD (Sweatt, 2003).

The medial temporal lobe contains many structures involved in learning and memory, including medial temporal cortex, the amygdala, the hippocampal formation, and the entorhinal, perirhinal, and parahippocampal cortices. The hippocampal formation contains the hippocampus proper, subiculum, and dentate gyrus. Experimental lesions of this area have revealed its essential function in the creation of new memories (Eichenbaum, 1999; Eichenbaum, et al., 1996). This role is further supported by the progression of neurodegeneration and corresponding deficits in learning and memory in AD.

The first clinical stages of AD are characterized by loss of episodic memory, slight difficulty with complex tasks, and limited spatial disorientation (Braak & Braak, 1998). These changes correlate chronologically with neurodegeneration in the entorhinal cortex and CA1, as well as increases in amyloid plaques and intracellular NFTs principally composed of hyperphosphorylated formed of the microtubule-associated protein tau (Spillantini & Goedert, 1998). Dysfunction and neurodegeneration

subsequently progresses to include the other areas of the hippocampus, the amygdala, and the limbic nuclei of the thalamus (Braak & Braak, 1998). In late stage AD, neurodegeneration has reached the cerebral cortex; and patients have a severe decline in cognition, a loss of episodic memory, and difficulty communicating (Braak & Braak, 1998).

Prior to the clinical manifestations of AD, cell death and other pathological changes, there are alterations to signal transduction and synaptic plasticity. As described in the previous chapters, apoE and the LDLR family are poised to play a role in cognitive changes that happen prior to the clinical onset of AD. While neither necessary nor sufficient to cause AD, apoE4 is well established as a disease risk factor (Corder, et al., 1993). The preclinical role of apoE in altering cognitive function is supported by studies associating apoE4 expression with alterations in learning (Baxter, et al., 2003) and memory (Bondi, et al., 1995; R. S. Wilson, et al., 2002), and other structural and functional abnormalities (Cohen, et al., 2001; Reiman, et al., 2004).

Experimental studies on the effects of apoE on learning and memory have focused on the use of genetically modified animals, namely apoE-deficient and apoE TR animals. The apoE-deficient mouse has been the subject of a number of behavioral studies. In several studies spatial memory is equivalent between apoE-deficient and murine apoE-expressing animals (Anderson, et al., 1998; Anderson & Higgins, 1997; Grootendorst, et al., 2005; Raber, et al., 2000). In others, spatial learning is impaired in the absence of apoE (Champagne, et al., 2002; Grootendorst, et al., 2001; Krzywkowski, et al., 1999; Masliah, et al., 1995; Oitzl, et al., 1997; Veinbergs & Masliah, 1999). These studies,

while yielding valuable information, emphasize the sensitivity of behavioral tests to genetic background, housing conditions, experimental designs, and other extrinsic factors.

For testing the effects of apoE isoform expression on learning and memory behavior, the apoE TR mouse is a valuable tool. Previous studies have shown significant differences in particular behaviors with apoE isoform expression. Female apoE4 TR mice have impaired spatial recognition versus apoE3 TR and apoE-deficient animals (Grootendorst, et al., 2005). While apoE3 TR female mice perform worse during water maze training, their spatial memory retention during the probe trial is on par with male apoE3 TR, apoE-deficient, and murine apoE-expressing animals. In contrast, both male and female apoE4 TR animals have impaired spatial memory retention (Grootendorst, et al., 2005). These impairments persist with age in female apoE4 TR animals (Bour, et al., 2008). Another study failed to replicate this deficit, likely due to differences in training protocol, but instead showed decreased performance in apoE4 TR mice is some, but not all, tests measuring anxiety behaviors (Villasana, et al., 2006).

In this study, I use young adult (3-5 month) male apoE TR mice to measure the effects of apoE isoform expression on learning and memory. To investigate the necessity of apoE in these behaviors, a parallel investigation of apoE-deficient and murine apoE-expressing animals was conducted. This study focuses on types of learning and memory behaviors known to be affected either by manipulations to the lipoprotein receptor system or in AD. Specifically, I have focused on working memory, associative learning, and

spatial memory with control experiments testing general locomotion, motor learning and coordination, sensorimotor gating, and nociception.

Methods

Animal maintenance and general behavioral assessment

ApoE2, apoE3 and apoE4 TR animals were obtained from a colony maintained at Taconic (Hudson, NY). ApoE knock-out and C57BL/6J animals were obtained from Jackson Laboratories (Bar Harbor, ME). All animals were fully backcrossed on a C57BL/6J background and bred in-house for at least 2 generations prior to selection for behavior. Animals were housed in a standard 12 hour light cycle and bred and maintained in accordance with the Vanderbilt University Institutional Animal Care and Use Committee protocol. Age-matched males were used for all behavioral testing. Animals were selected for behavioral testing at 3 months of age. Animals progressed through the behavioral tests from least to most invasive: open field, rotorod, novel object recognition, water maze. A second cohort of animals were housed and tested in the Murine Neurobehavioral Laboratory at University of South Florida. After confirming that the general behavior profile of animals housed at this location did not differ from those housed at Vanderbilt University, these animals were utilized for pre-pulse inhibition, acoustic startle, fear conditioning, and shock threshold.

General activity was measured by the open field task. This task consists of a 27 cm by 27 cm chamber containing 16 photoreceptor beams on each side of the chamber.

Locomotion measurements were analyzed by the computer-operated animal activity session (Med Associates, St. Albans, VT) during the 15 minute session. To assess motor coordination and motor learning, animals were subjected to the accelerating rotating rod (rotorod) task (Ugo Basile, Comerio Italy). In each 300 second session, the rod accelerated from 4 to 40 rpm; animals were given 4 sessions per day for 2 consecutive days and latency to fall was measured.

Learning and memory behavior assessment

Working memory: The novel object recognition task assesses working memory in mice. During training, two distinct objects were placed in the 49.5 x 33 x 28 cm arena and the animal was allowed to explore for three 5-minute trials with a 5 minute inter-trial interval. For the test trial (5 minutes), one of the previously explored objects was replaced with a novel object. The objects and arena were cleaned with 70% ethanol between each trial to eliminate olfactory cues. The test was quantified in a genotype-blind fashion by the experimenter. The ability of the animal to distinguish between the novel and familiar objects was expressed as the discrimination index, or the percentage of time exploring the novel object versus the total object exploration time. Object exploration was defined as the nose of the animal coming within 2 cm of the object.

Sensorimotor gating: Pre-pulse inhibition was measured by placing the animal in an isolation chamber on top of a high sensitivity weight transducer to measure movement (Panlab, Barcelona Spain). After a five minute habituation with background white noise at 65 dB, the animals were presented with seven different auditory stimuli in a pseudo

random order: 1) a 120 dB pulse at 8000 Hz lasting for 40 ms; 2-6) a 20 ms pre-pulse at 74, 78, 82, 86, or 90 dB followed by the 120 dB pulse; 7) no pulse. Each animal received 6 blocks of these seven trials, and peak startle amplitude within 70 ms of the pulse was analyzed. Pre-pulse inhibition was calculated as the amount of reduction of the startle amplitude with each of the pre-pulse dB levels versus the startle amplitude to the pulse alone.

Associative learning: The fear conditioning was conducted in an apparatus consisting of a 250x250x250 mm plexiglass chamber with a metal grid floor capable of delivering a mild foot shock housed within a soundproof chamber with white noise generated by a small fan. Movement was measured by a high-sensitivity weight transducer (Panlab, Barcelona Spain). During conditioning, animals were allowed to explore the chamber under white light for 2 minutes prior to the onset of the conditioned stimulus (CS), an 85 dB tone with a 30 second duration. The unconditioned stimulus (US), a 0.5 mA foot shock, was delivered during the last 2 seconds of the CS. A second CS-US pairing occurred 90 seconds after the first, and the mouse was returned to the home cage after a total time of 7 minutes. Contextual memory tests were performed both 1 and 24 hours after training. Animals were placed back into the conditioning chamber for 3 min and freezing behavior was measured. Freezing was defined as a lack of movement for 2 seconds.

Shock threshold was determined by returning the animals to the fear conditioning chamber. Foot shock intensity began at 0.05 mA and increased in 0.1 mA intervals from 0.1 mA every 30 seconds to a maximum of 0.8 mA. The shock intensity necessary to

induce flinching, jumping or vocalization was recorded and the experiment was terminated upon vocalization.

Spatial learning: Testing of spatial learning and memory was conducted by using the Morris hidden platform water maze test. A 91.5 cm diameter plastic Nalgene pool was filled with room temperature (~22°C) and made opaque with white non-toxic tempera paint. Prominent extra-maze cues were present around the room. For training, mice were placed in one of four starting locations along the pool wall and allowed to swim until finding an 8 cm x 8 cm Plexiglas platform submerged 1 cm below the water surface or a maximum of 60 seconds. Upon finding the platform, or being placed on the platform by the experimenter at the end of the 60 second trial, mice remained on the platform for 20 seconds before being returned to their home cage. Latency to reach platform, distance traveled to platform and swim speed was obtained using automated video tracking software (ANY-Maze, Stoelting). Mice were trained with four trials per day (1 hr inter-trial interval) for eight consecutive days. At one hour and 24 hours following the last training session a probe trial was performed. The probe trial consisted of a 60 second free swim with the submerged platform removed. Number of crossings of the platform location and percent time spent in the target quadrant was calculated for each probe trial. Visual acuity was measured at the end of the probe trials by latency to find a flagged submerged platform in a novel location.

Statistics

Data was analyzed between wild-type and apoE-deficient animals with a two-tailed t-test and across apoE TR genotypes using one-way ANOVA with Bonferroni's post hoc tests.

Data is expressed as mean ± SEM, percent of apoE-deficient where appropriate. Significance was set at $p<0.05$ for all tests.

Results

General behavior performance

In this study, I hypothesized that specific apoE isoform expression will differentially alter performance of learning and memory tasks, in particular those tasks that require proper hippocampus function such as spatial learning and contextual associative learning. These tests require complex behavior responses and depend not only on hippocampus function but the ability of an animal to gather sensory information, properly integrate this information, and perform the desired behavior. To control for any differences that apoE isoform expression or apoE-deficiency may have on these abilities, I began this study with a series of general behavioral assessments.

There have not been any previously reported changes in general locomotion with apoE expression in apoE TR mice. General locomotive behavior was quantified by the open field task. During the 15 minute test, there were no differences in total distance traveled between any of the apoE TR animals or between apoE-deficient and wild-type animals (figure 17A, WT vs E0 t-test $p= 0.3070$, E2 vs E3 vs E4 ANOVA $p=0.1304$). Capitalizing on the tendency of mice to avoid open, well-lit areas while exploring a novel environment, we used the ratio of distance traveled in the center of the open field arena to total distance traveled as a rough measurement of anxiety. Highly anxious rodents will

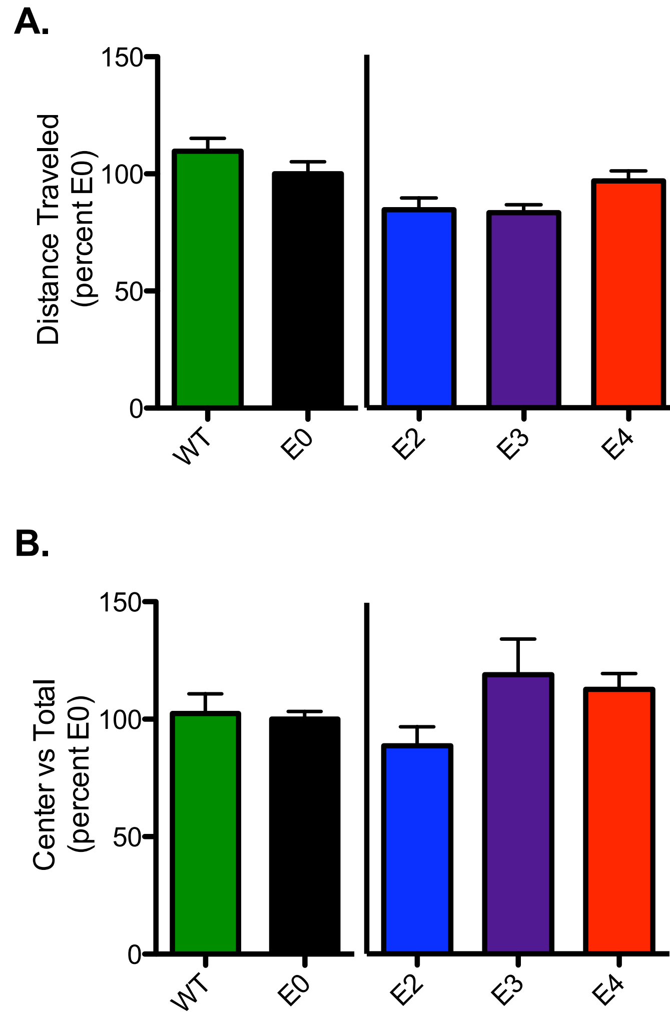


Figure 17: General locomotion is unaltered by absence of apoE or apoE isoform expression

A. Distance traveled during 15 minute open field session, expressed as percent of apoE-deficient (E0). B. Ratio of distance traveled in center portion of open field arena to total distance traveled, expressed as percent of E0. WT=wild-type n=8; E0=apoE-deficient n=12; E2=apoE2 TR n=12; E3=apoE3 TR n=8; E4=apoE4 TR n=12. Data expressed as mean ± SEM, percent of E0.

often preferentially avoid the center portion of the arena. There were no significant differences in this ratio between apoE-deficient and wild-type animals, or between apoE2 TR, apoE3 TR, and apoE4 TR animals (figure 17B WT vs E0 t-test p=0.7572, E2 vs E3 vs E4 ANOVA p=0.0697).

Testing of spatial and associative learning requires proper motor coordination. Therefore, we tested the animals using the accelerating rotating rod (rotorod). Animals were placed on the rotating rod, which accelerate from 4 revolutions per minute (RPM) to 40 RPM over a 5 minute period, and latency to fall was measured. All animals were able to learn the task after four trials per day for two consecutive days (figure 18A,C). However, apoE3 TR animals performed worse than apoE2 TR and apoE4 TR animals on early trials resulting in an overall reduced average latency (figure 18C,D ANOVA p=0.0065). While this may indicate a deficit in motor learning, the animals do reach a similar performance on the final training session as the other groups.

Effect of apoE isoform expression on working memory

Working memory was tested using the novel object recognition task. During the test trial, time exploring the novel object and the familiar object was recorded These times were used to calculate the discrimination index, or the time exploring the novel object versus the total object exploration time.

All groups showed a preference for the novel object as shown by a discrimination index greater than 50 (figure 19A). There were no significant differences in discrimination index between apoE-deficient and wild-type animals, or between apoE2

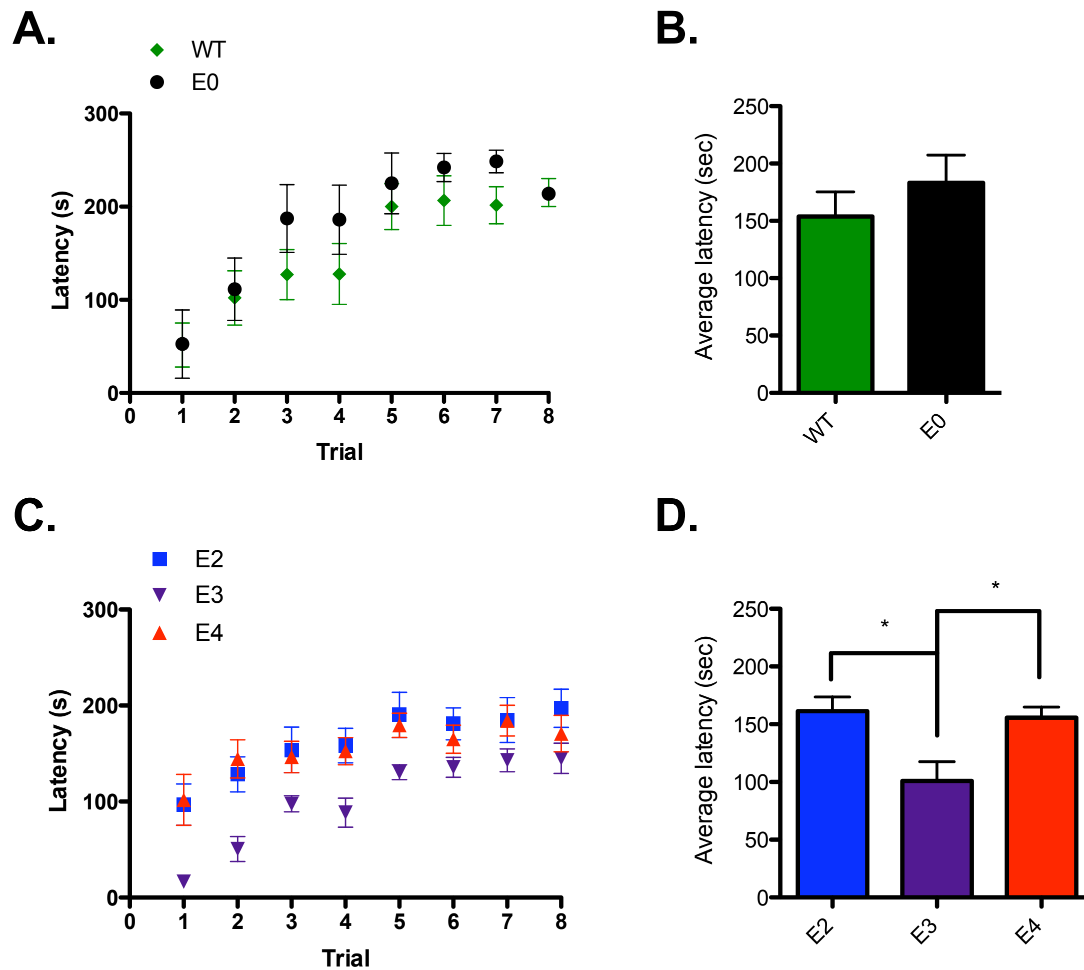


Figure 18: Performance on accelerating rotorod
A. Latency to fall from rotating rod for each trial for wild-type and apoE-deficient animals. B. Average latency over all trials.
C. Latency to fall from rotating rod for each trial for apoE2, apoE3, and apoE4 TR animals. D. Average latency over all trials
WT=wild-type n=6; E0=apoE-deficient n=6; E2=apoE2 TR n=7; E3=apoE3 TR n=9; E4=apoE4 TR n=6. Data expressed as mean ± SEM. *p<0.05, ANOVA with Bonferroni's post-test

TR, apoE3 TR, and apoE4 TR animals (figure 19A Wt vs E0 t-test p=0.8796, E2 vs E3 vs E4 ANOVA p=0.1984). Total object exploration time was also not significantly different between apoE-deficient and wild-type animals (figure 19B, t-test p=0.5489). There was greater variability in total object exploration time in apoE TR animals; apoE4 TR animals had a lower average total object exploration time than apoE2 TR and apoE3 TR animals (figure 19B, ANOVA p=0.0097).

Effect of apoE isoform expression on associative learning

As hippocampal function and associative learning is highly dependent on sensory input, I first tested for normal sensory response and sensorimotor gating. Auditory function was evaluated by measuring startle response to a 40 ms, 120 dB tone. The peak amplitude of startle response within 70 ms of the tone was compared to the startle response from an identical time block not preceded by a tone. As expected, all groups showed a significant startle response (figure 20). There was no significant difference in startle response between wild-type and apoE-deficient animals (figure 20A, t-test p=0.0991). Notable, however, is the reduction in overall startle amplitude in apoE2 TR animals as compared to apoE3 TR and apoE4 TR animals (figure, 20A ANOVA p=0.0039). The apoE2 TR does show significant startle behavior to the tone (t-test pulse versus no pulse, p=0.0070), which indicates that these animals can hear and react to the tone. However, the lower startle amplitude may reflect a deficit in sensorimotor gating.

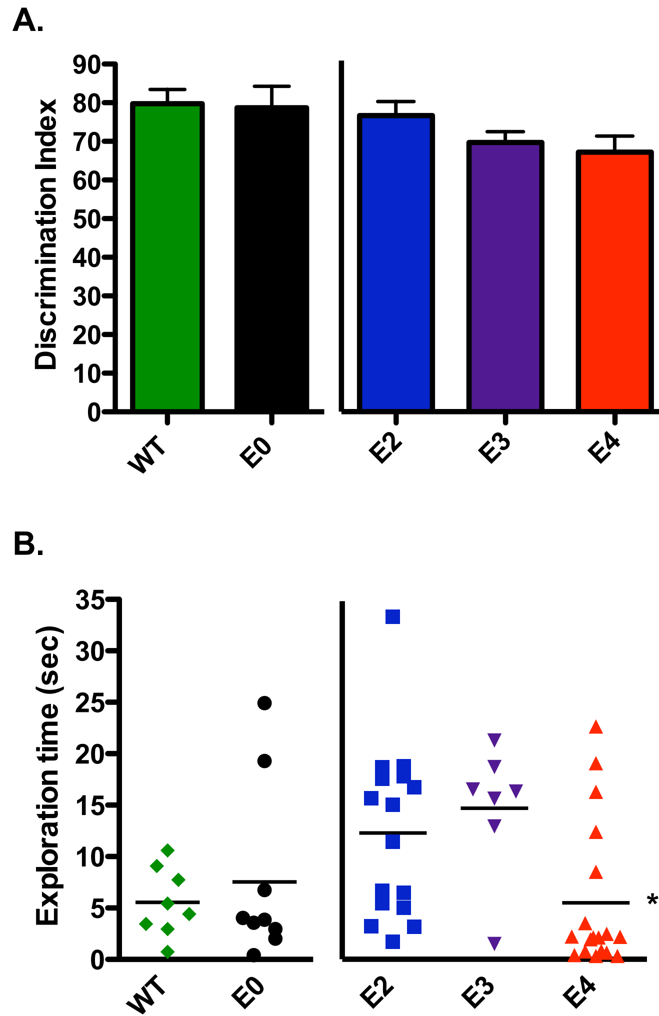


Figure 19: Working memory performance in the novel object recognition task
A. Working memory behavior as measured by discrimination index - time exploring novel object versus total object exploration time - during five minute test trial. B. Total object exploration time. WT=wild-type n=8; E0=apoE-deficient n=9; E2=apoE2 TR n=12; E3=apoE3 TR n=8; E4=apoE4 TR n=12. Data expressed as mean ± SEM.

When a weaker stimulus is presented prior to a stronger one, the nervous system can temporarily adapt to reduce the reaction to the strong stimulus. This phenomenon, called pre-pulse inhibition (PPI), is impaired by conditions that alter sensorimotor gating, the most classic example being schizophrenia (D. Braff, et al., 1978; D. L. Braff, et al., 2001).In mice, PPI is induced when a lower intensity pre-pulse tone of 74-90 dB is given 100 ms prior to the 120 dB test tone. PPI normally increases with increased pre-pulse decibel. We observed this typical pattern in our wild-type animals, confirming our PPI protocol (figure 20B). ApoE-deficient animals also showed this typical behavior, albeit with some variability, but these differences were not significant (figure 20B). The maximum PPI measured was equivalent in both the apoE2 and apoE3 TR animals; apoE4 TR animals reached a lower maximum PPI but this difference was not significant.

Classical associative learning and memory is measured in the mouse by the fear conditioning paradigm (Fanselow, 1980; Fanselow & Tighe, 1988). Animals were placed in a novel environment—a plexiglass cage with an electrified floor within a sound-attenuating chamber—and allowed to explore for 2 minutes. Subsequently animals were given two pairings of the unconditioned stimulus (US), a mild foot shock, and the conditioned stimulus (CS), an 85 dB tone.

With this test, animals must make associations between the context, the CS, and the US. Fear response is measured by the percent time spent displaying the typical freezing posture, defined for this study as a lack of movement for 2 seconds. All groups demonstrated this freezing behavior which increased with each CS-US pairing during training, indicating all animal groups correctly respond to the US (figure 21A).

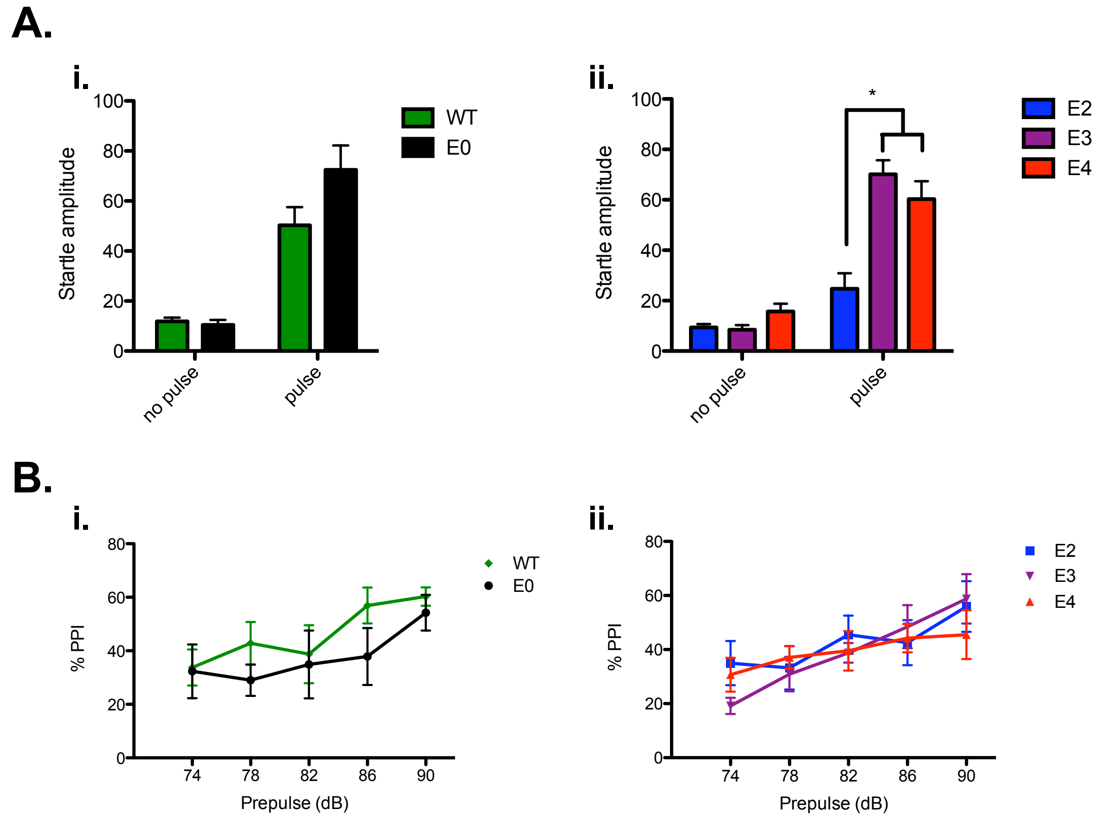


Figure 20: Startle amplitude and pre-pulse inhibition

A. Amplitude of startle response in the absence (no pulse) or presence (pulse) of 120 dB tone for wild-type (C57BL/6J) and apoE-deficient (i) and apoE2, apoE3, apoE4 TR (ii) animals. B. Percent inhibition of startle response to pulse by a preceding pre-pulse for wild-type and apoE-deficient (i) and apoE2, apoE3, apoE4 TR (ii) animals. WT=wild-type n=7; E0=apoE-deficient n=4; E2=apoE2 TR n=9; E3=apoE3 TR n=8; E4=apoE4 TR n=9. Data expressed as mean ± SEM. *$p<0.05$, ANOVA with Bonferroni's post-test.

Proper formation of associative memories was tested by re-exposing animals to the context, the training box without the CS or US, one hour after training. This testing of contextual associative memory requires proper functioning of both the hippocampus and amygdala. When re-exposed to the context one hour after training, we found equivalent freezing behavior in both the wild-type and apoE-deficient animals as well as the apoE2, apoE3, and apoE4 TR animals (figure 21B WT vs E0 t-test p=0.2742, E2 vs E3 vs E4 ANOVA p=0.2046). Long term retention of this memory was measured by re-exposure to the context 24 hours post-training. During this test, percent freezing was significantly different between apoE2 TR and apoE4 TR mice (figure 21C, ANOVA p=0.0023). Percent freezing was not significantly different between wild-type and apoE-deficient animals (figure 21C, t-test P=0.9463)

Differences in nociception could complicate the interpretation of the fear conditioning results since increased or reduced sensitivity to the foot shock would impact association of US to context and CS. In order to ensure there were no such nociceptive differences, the threshold of foot shock necessary to elicit flinching, jumping, or vocalization was measured.

After at least one week recovery from fear conditioning, animals were placed back into the experimental chamber. A one-second foot shock was given every thirty seconds starting at 0.05 mA and increasing in 0.1 mA intervals from 0.1 to 0.8 mA or until vocalization. At low shock intensities, both wild-type and apoE3 TR animals had a tendency to flinch earlier than apoE-deficient or apoE2 and animals, respectively (figure 22A, WT vs E0 t-test p=0.0042, E2 vs E3 vs E4 ANOVA p=0.0343). In contrast, there

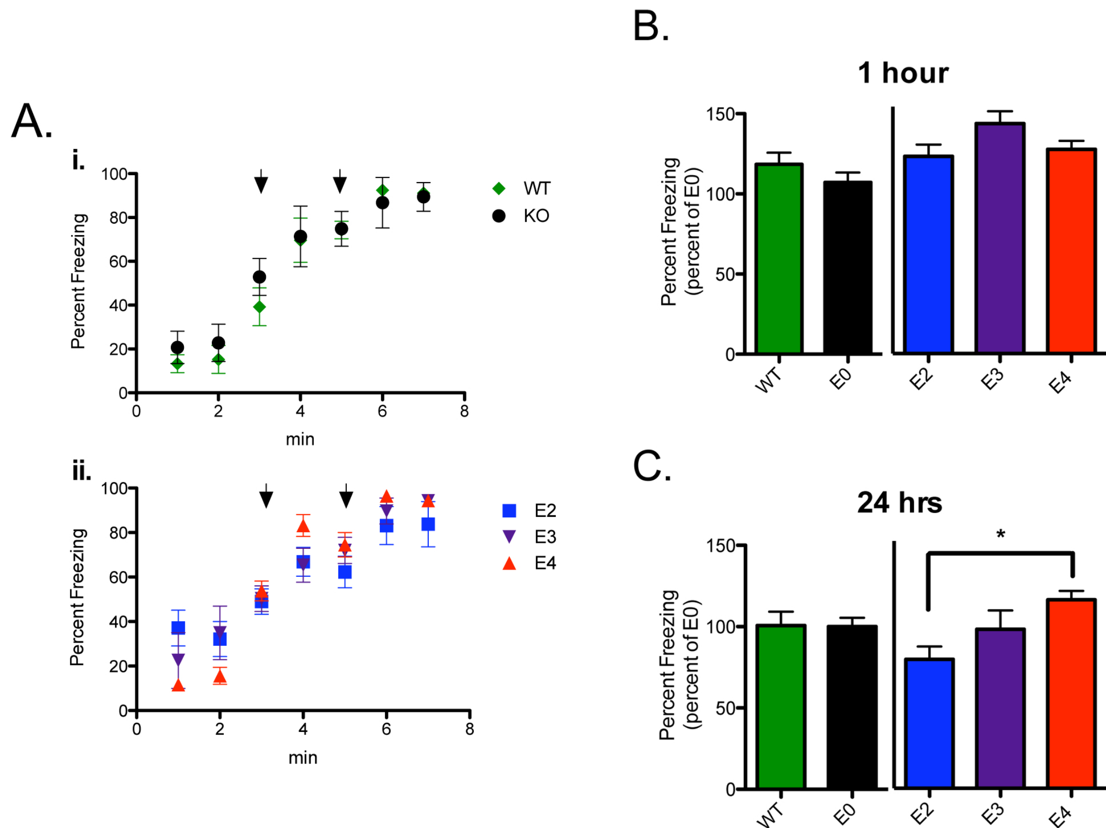


Figure 21: Associative memory performance in fear conditioning test
A. Percent time spent freezing during training for fear conditioning test. Arrows indicate foot shocks for wild-type and apoE-deficient (i) and apoE2, apoE3, apoE4 TR (ii) animals. B. One hour and C. 24 hour contextual testing. Percent time displaying freezing behavior when re-exposed to training context. WT=wild-type n=7; E0=apoE-deficient n=4; E2=apoE2 TR n=9; E3=apoE3 TR n=8; E4=apoE4 TR n=9. Data expressed as mean ± SEM, percent of E0. $*p<0.05$, ANOVA with Bonferroni's post-test.

were no differences in the shock threshold required to induce jumping or vocalization (figure 22B,C). Importantly, the foot shock amplitude used in fear conditioning is in the middle of this range (0.5 mA), showing that there are similar nociceptive responses regardless of apoE isoform.

ApoE isoform expression and spatial memory

To determine if apoE isoform expression affects spatial learning, the Morris hidden platform water maze test was used. Animals were trained to find the hidden platform using four trials per day for 8 consecutive days. All animals were able learn the task and utilize spatial memory to find the hidden platform without any effect of apoE isoform (figure 23). As a control, visual acuity was by measuring latency to find the platform when identified with a conspicuous flag. All groups were able to find the visually identified platform; there were no significant difference between apoE TR groups and no group averaged more than 20 seconds to find the platform (figure 23B). In addition, there were no significant differences in overall swim speed (figure 23C).

One hour after training on day 8, the platform was removed for a probe trial to test short-term spatial memory. Animals that have learned the task will also spend more time in the target quadrant than the other three quadrants. During the 60 second probe trial, all animal groups spent significantly more time in the target quadrant (TQ) than the other quadrants (figure 24A, ANOVA WT $p<0.001$, KO $p<0.001$, E2 $p=0.0029$, E3 $p<0.001$, E4 $p<0.001$).

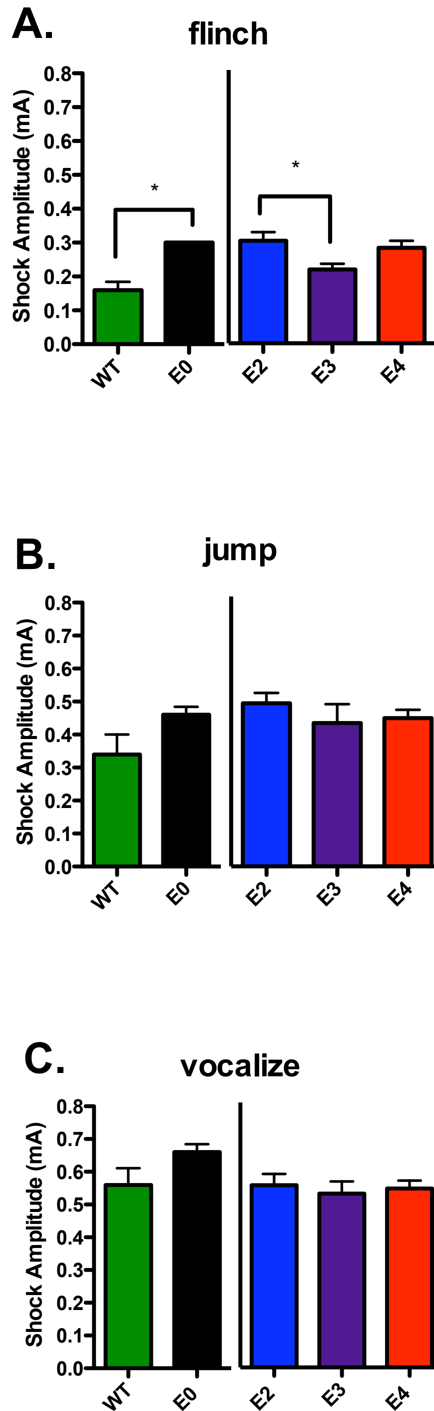


Figure 22: Foot shock threshold

Foot shock intensity required to induce flinching (A), jumping (B), or vocalization (C) in wild-type and apoE-deficient and apoE2 TR, apoE3 TR, and apoE4 TR animals. WT=wild-type n=7; E0=apoE-deficient n=4; E2=apoE2 TR n=9; E3=apoE3 TR n=8; E4=apoE4 TR n=9.. Data expressed as mean ± SEM.

Twenty-four hours after the last training session, another probe trial was given to test for long-term retention of spatial memory. At this time, percent time in target quadrant was significantly greater than at least one other quadrant for all mice except apoE4 TR (figure 24B). Wild-type animals spend significantly more time in the target quadrant than any other quadrant (figure 24B, ANOVA WT $p=0.002$). ApoE-deficient animals spend significantly more time in the target quadrant than two of the three other quadrants (figure 24B, ANOVA E0 $p<0.001$).

ApoE3 TR animals also spend significantly more time in the target quadrant than any other quadrant during the 24 hour probe trial (figure 24B, ANOVA $p=0.0017$). In contrast, the time spent by apoE2 TR animals is only significant from the opposite quadrant (figure 24B, ANOVA $p=0.0448$). ApoE4 TR animals revealed no significant differences in percent time in the target quadrant from any other quadrant (figure 24B, ANOVA $p=0.0517$).

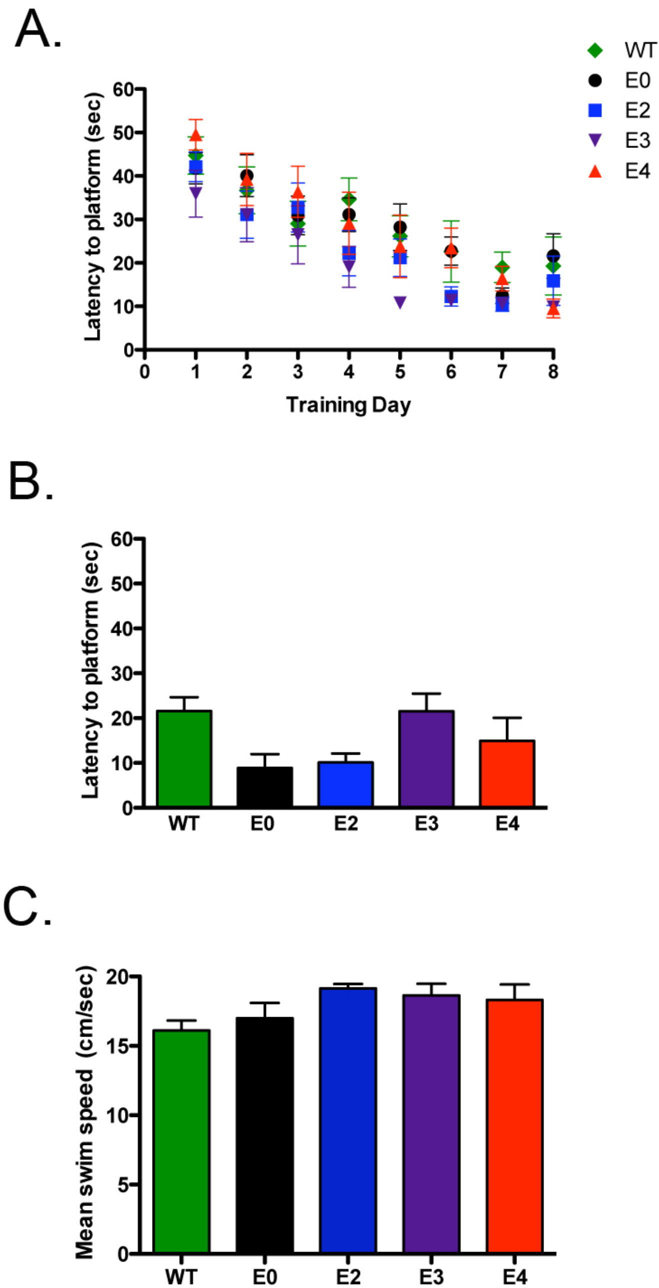
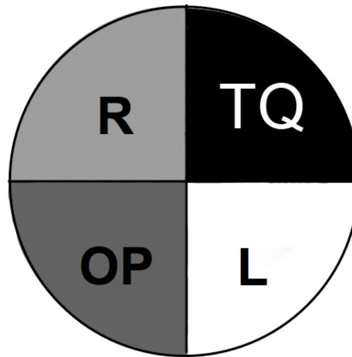
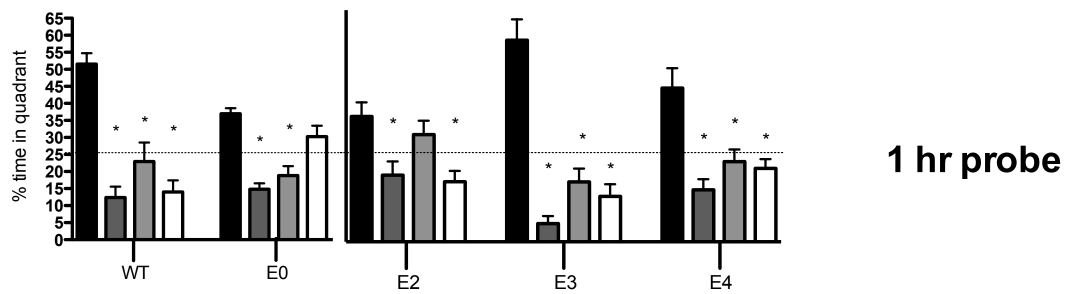


Figure 23: Water maze training

A. Animals were trained with four trials per day (1 hour inter-trial interval) for 8 consecutive days. Average latency to find submerged hidden platform each day. B. Latency to find submerged platform visually identified with flag. C. Mean swim speed. WT=wild-type n=6; E0=apoE-deficient n=9; E2=apoE2 TR n=9; E3=apoE3 TR n=6; E4=apoE4 TR n=8 Data expressed as mean ± SEM.



A.



B.

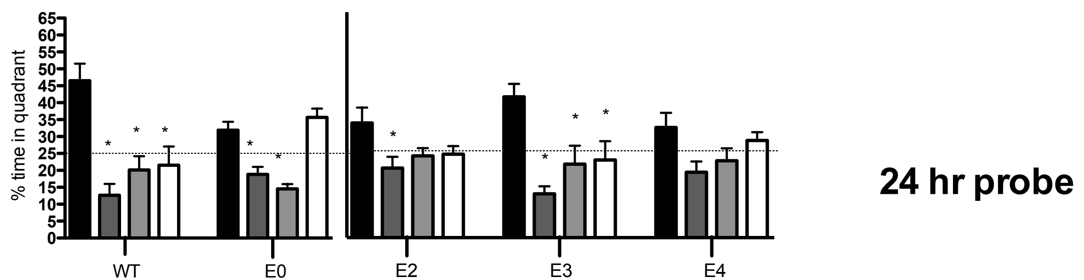


Figure 24: Spatial learning performance in water maze task
Diagram indicates quadrant positions: TQ = target quadrant (platform location), O=opposite, R=right, L=left.
A. Percent time in quadrant during probe trial consisting of a 60 second free swim without platform performed one hour after conclusion of training on day 8.
B. Percent time in quadrant during probe trial 24 hours after conclusion of training. WT=wild-type n=6; E0=apoE-deficient n=9; E2=apoE2 TR n=9; E3=apoE3 TR n=6; E4=apoE4 TR n=8 Data expressed as mean ± SEM. *$p<0.05$ versus TQ for each group, ANOVA with Bonferroni's post test

Discussion

These behavioral studies were designed to test the hypothesis that specific apoE isoform expression will elicit equally specific effects on cognitive ability, in particular learning paradigms dependent on normal hippocampus function such as spatial learning. This hypothesis evolved from the finding that alterations to lipoprotein receptors, specifically apoER2 and VLDLR, can alter spatial learning and memory and associative learning (S. Qiu, Korwek, Pratt-Davis, et al., 2006; Weeber, et al., 2002). As a ligand for these receptors and a modulator of synaptic function, apoE may also affect the complex processes underlying learning and memory behavior in the adult mouse. ApoE has the potential to alter cognitive function based on specific isoform expression due to its interactions with known signaling pathways in the hippocampus (for review see (S. Qiu, Korwek, & Weeber, 2006)). To date, there are numerous studies linking apoE isoforms to changes in human cognitive function (Bennett, et al., 2005; Caselli, et al., 2001; Lind, et al., 2006) and mouse behavior (Bour, et al., 2008; Grootendorst, et al., 2005; Hartman, et al., 2001; Villasana, et al., 2006). In this study, I addressed both the necessity of apoE in specific learning and memory behaviors as well as the role of human apoE isoforms in modulating such behavior. To do this, I compared the behavioral profile of apoE2, apoE3 and apoE4 TR animals as well as apoE-deficient and murine apoE-expressing animals (wild-type) in a single study, thus separating the effects of the presence of human apoE from the consequences of the absence of murine apoE.

Behavioral studies are highly sensitive to non-experimental extenuating factors such as housing, handling, genetic background, maternal care, and external stimuli. The

design of my study aims to minimize this inter-lab variability by testing all three lines of apoE TR mice at the same time to isolate apoE isoform-dependent changes in learning and memory. I focused on three types of memory: working, associative, and spatial. These types of memory are usually impaired in early AD and may be susceptible to alterations in signal transduction pathways that rely on apoE signaling. Moreover, I can compare the non-hippocampal working memory test of novel object recognition to the hippocampus-dependent spatial learning paradigm of the hidden platform water maze test and contextual fear conditioning.

This study both complements and expands upon previous studies of the same apoE TR animals. I confirmed a lack of changes to general locomotion in male animals (Grootendorst, et al., 2005) and as well as previously reported reductions in rotorod latencies in apoE3 TR animals (Villasana, et al., 2006). Other than the difference in rotorod latencies, I did not find any other general behavioral differences in the apoE TR. There were no significant differences in motor learning, nociception, sensorimotor gating, or auditory response. The apoE2 TR animals did have less response overall startle response to the auditory stimulus. However, their overall startle response to the stimulus was greater than their response in the lack of auditory stimulus. Combined with the lack of changes in PPI, it is likely that the overall reduction in startle amplitude in apoE2 TR animals is not indicative of a sensorimotor gating deficit sufficient to impact other behavioral tests rather an altered reflex response.

Our short-term working memory is essential for our ability to function in our day-to -day lives. Without it we would struggle with remembering why we walked into a room,

who we wanted to call when we picked up the phone, or what we had for breakfast—or if we had breakfast at all. AD patients struggle with deficits in working memory which significantly impairs function and quality of life (Bailey, et al., 2004). I sought to investigate the role of apoE isoform on this type of memory in apoE TR animals with the novel object recognition task. The novel object recognition task capitalizes on the natural inclination to preferentially explore new additions to their environment over the familiar. While widely regarded as hippocampus independent, this activity is linked to temporal lobe structures such as the perirhinal cortex (Morris, et al., 1982). There were no significant differences in working memory as measured by novel object recognition. Total object exploration time did vary with apoE isoform. However, the constant preference to spend more time exploring the novel object, as evidenced by a discrimination index greater than 50, makes it unlikely that these changes in exploration time are masking a working memory deficit. While AD patients do experience working memory deficits, the apoE TR mice do not develop AD-like pathologies as seen in other mouse models of the disease. Thus, it is reasonable to assume that the apoE TR animals will not recapitulate all of the behavioral hallmarks of AD but rather allow us to isolate those behaviors that are specifically modulated by apoE isoforms.

The hippocampus is central to muti-modal processing, incorporating direct and indirect sensory inputs, modulatory inputs from the brain stem, and cortical connectivity as it coordinates associative learning and memory consolidation (Eichenbaum, 1999). These abilities are impaired by hippocampal lesions or neurodegeneration leading to dementia (Mayes, et al., 2004; Rawlins & Tanner, 1998; Weniger, et al., 2004). Using the

fear conditioning paradigm, which has been well validated in mice (Phillips & LeDoux, 1992), I tested the effect of apoE isoform on associative learning and memory. Associative learning varied with apoE isoform, as apoE2 TR mice showed a significant difference in freezing to the fear conditioning context 24 hours after training as compared to apoE4 TR mice. This is a specific change in long-term retention of associative memory as both groups showed equivalent response to both the fear conditioning training and testing of short-term contextual associative memory. This finding means that possession of apoE2 or apoE4 is sufficient to alter long-term associative memory without alterations to learning or short-term memory. The absence of changes in apoE-deficient or apoE3 TR animals suggests that the effects seen in the presence of apoE2 or apoE4 are due to active modulation of cognitive processes by these isoforms.

Creating a spatial map of ones environment invokes the hippocampus; spatial learning and memory is especially sensitive to hippocampal lesions (Logue, et al., 1997; Morris, et al., 1982). The formation of these and other new long-lasting memories requires a highly orchestrated set of biochemical changes, including protein synthesis-dependent changes within neurons that generate a persistent biochemical signal . Spatial memory in particular also appears to be sensitive to proper lipoprotein receptor signaling. Studies of apoER2 and LRP deficiency show defects in hidden platform spatial learning (May, et al., 2004; Weeber, et al., 2002).

In this study, long term spatial memory retention is decreased in apoE4 TR animals. ApoE4 TR animals did not show significantly more target platform crossings than any other quadrant during the probe trial testing spatial memory 24 hours after the completion

of water maze training. Previous studies have found spatial memory deficit in female apoE4 TR (Bour, et al., 2008; Grootendorst, et al., 2005), and this study expands this deficit to male mice. The effect of apoE2 on spatial memory is less clear, as these animals do spend significantly more time in the target quadrant than the opposite quadrant. Expansion of the search radius by an animal often results in time being spent in the left and right quadrants, so it is possible that these animals do not have impairments in long term spatial memory retention. Additional testing with a second cohort of animals will confirm spatial memory performance in apoE2 and apoE4 animals and reveal possible differences in search strategy.

Likewise, apoE-deficient animals in this study also spend significantly more time in the target quadrant than both the opposite and right quadrants. While a detailed analysis of quadrant time is not available for all previous studies in the literature, there is a prescient for both impairments and no impairments in spatial memory in apoE-deficient mice. Findings of impaired spatial memory attributable to procedural differences in training protocols, for instance massed training sessions instead of spaced (Oitzl, et al., 1997), or sex or age differences in the mice used in those studies (Masliah, et al., 1997; Veinbergs, et al., 1999) versus the young adult male mice used in this study. In contrast, other studies support the findings of this study (Anderson, et al., 1998; Anderson & Higgins, 1997; Bour, et al., 2008), demonstrating the subtlety of behavioral testing for learning and memory. The absence of a spatial memory deficit in the apoE3 TR animals also emphasizes the specificity of apoE isoform dependent modulation of learning and memory.

The two tests of hippocampus-dependent learning and memory in this study, fear conditioning and water maze, yielded different results. ApoE4 TR animals are not deficient in associative learning but are deficient in spatial memory. The finding that the effects of apoE isoform on learning and memory vary with behavioral paradigm demonstrates both the subtlety and specificity of apoE isoform-dependent modulation. Such small changes in behavior are not uncommon with alterations to the pathways surrounding apoE receptors. For example, selenoprotein P-1 (Sepp1) deficient mice display normal associative learning and memory, but impaired acquisition of spatial learning (Peters, et al., 2006). As Sepp1 binds to and signals through apoER2, much like apoE, this supports our finding that alterations to the modulators of the lipoprotein receptor system can have subtle yet notable implications on learning and memory.

While tests of associative and spatial memory do tap into similar neurological pathways, each recruits different brain regions with varying levels of complexity. Water maze spatial learning is severely impaired with hippocampal lesions (Logue, et al., 1997); performance is also impaired by lesions of the medial septum/diagonal band, the entorhinal/peririhinal cortex, or by treatment with cholinergic and glutamate receptor antagonists (Decker & Majchrzak, 1992; McNamara & Skelton, 1993; Nagahara, et al., 1995; Nilsson & Gage, 1993). This task requires the formation and refinement of a spatial map over a period of days, as well as the ability to retain the memory of that map. The complexity of this task contrasts the more straight-forward contextual fear conditioning. Fear conditioning requires a different set of sensory and motor abilities, and capitalizes on an animal's natural response to fear-inducing stimuli. Much like water

maze, fear conditioning utilizes the hippocampus but also involves the amygdala, subiculum, cingulate cortex, prefrontal cortex, perirhinal cortex, sensory cortex, and medial temporal lobe (Eichenbaum, et al., 1996; Fanselow & Kim, 1994; LeDoux, et al., 1990; Logue, et al., 1997; Phillips & LeDoux, 1992; Squire & Zola, 1996). Thus, the variation seen in the effect of apoE isoforms on learning and memory based on task may stem from the involvement of particular brain regions and the specific effects that apoE isoforms have on function. Such differences are not unprecedented, as apoE isoform-dependent effects on synaptic plasticity vary widely between the perforant path (Trommer, et al., 2004) and area CA1 (chapter III) in the hippocampus.

The behavioral profile of the apoE TR animals combined with the lack of major deficiencies in apoE-deficient mice supports the view that apoE and its receptors are modulators of learning and memory. Absence of apoE does not turn “off” synaptic function nor does the presence of apoE turn it “on”. The synaptic mechanisms altered by apoE isoforms are beginning to be elucidated (see chapters 2 and 3). Making a direct connection between synaptic plasticity and behavior is difficult at best. However, the findings presented here support the hypothesis that apoE isoform expression plays an active role in the modulation of cognitive function and establishes a basal behavioral profile for apoE TR animals that will be useful in future studies.

CHAPTER V

DISCUSSION

During memory formation, the integration of incoming somatosensory inputs with pre-existing experience determines the importance of the combined signal and influences the production of new memories. However, ultimate control over these processes is performed at the level of neuronal modulation. Changes in the expression of specific cell surface receptors, sensitivity to neurotransmitter release or activation of transmitter-dependent signal transduction cascades allows for surprising flexibility and control over synaptic plasticity and cognitive ability. While a multitude of molecules contribute to this process, those with the potential to modify synaptic function to the greatest extent are proteins that can play a role in all of the molecular changes listed above. The lipoprotein receptor system, and its ligand apoE, firmly fit into this category of important modulatory proteins. Dysfunction or altered function within this receptor system are linked to several disease states and altered cognitive function. Yet little is known about how different human isoforms of apoE signal through their receptors, if isoform variation changes signal transduction capability, or the consequences of these differences in synaptic plasticity and cognitive ability.

ApoE and its highly conserved family of receptors appear to have been co-opted from simple cholesterol transport to regulate neuronal function. Poised on the surface of neurons, the LDL receptor family mediates transport of apoE and other ligands across the

cell membrane. Among these other ligands is one in particular that has the ability to induce cellular signaling sufficient to alter synaptic function: the extracellular matrix protein reelin. Well characterized in the literature, reelin signaling through lipoproteins receptors modulates both neuronal positioning during development and neuronal signaling in the adult brain. Our understanding of reelin signaling and its importance in normal CNS function gives valuable insight into the potential actions of other ligands known to signal through this lipoprotein receptor family, such as apoE.

The coupling of signaling pathways initiated by reelin signaling through lipoprotein receptors to physiologic changes in the hippocampus supports the hypothesis that other ligands of the lipoprotein receptor family have the capability to play a role in neuronal physiology as well. The ability for apoE to bind to all members of the family of lipoprotein receptors initiated the present study investigating the potential for apoE to act as an isoform-specific signaling ligand in the hippocampus. For this study, I utilized the tools developed from previous investigations of the effect of reelin on synaptic plasticity and signal transduction as a primer to investigate the effects of apoE. The experimental design of my study allowed for multiple lines of comparison including:

1) Direct comparison of all three endogenous human apoE isoforms in apoE TR mice.

2) Comparison of the signaling effects of exogenous apoE application to reelin mediated signaling.

3) Comparison between the effects of chronic apoE expression and acute apoE exposure.

Thus, the experimental design of this study was specifically considered to create a more complete picture of lipoprotein receptor signaling in the hippocampus, establish a

baseline for future studies of apoE signaling and begin to dissect out potential mechanisms for isoform specific changes in neuronal function.

Implications of acute apoE application

In hippocampus area CA1, either chronic apoE isoform expression or acute apoE isoform exposure produces the same response to a theta-burst protocol for inducing LTP. The presence of apoE4 results in the greatest LTP induction while the presence of apoE2 results in the least; both the presence of apoE3 and apoE-deficiency are intermediary to these two extremes (figures 5 & 10). Initially this appeared to indicate that apoE acts as an isoform-specific signaling molecule, and that the two delivery methods have equivalent effects. As the enhanced LTP induction in the presence of apoE4 looked remarkably similar to that seen in the presence of exogenous reelin (Weeber, et al., 2002), it was reasonable to interpret that apoE4 may be increasing NMDA receptor function similar to reelin and supported the hypothesis that an isoform of apoE could have a physiologic response similar to reelin (S. Qiu, Zhao, et al., 2006). It was in the investigation of this hypothesis that the differences between chronic apoE expression and acute apoE exposure became apparent.

With the apoE4-induced enhancement of LTP induction, it was hypothesized that acute apoE4 application would also increase NMDAR-mediated field potentials, again similar to that seen with reelin application. Instead, both apoE2 and apoE4 application reduce NMDAR-mediated field potentials (figure 6). This change in NMDA receptor function could be due to changes in phosphorylation. In fact, activation of lipoprotein

receptors by reelin alters tyrosine phosphorylation of NMDA receptors, which correlates nicely with the changes seen in LTP induction and NMDAR currents (S. Qiu, Zhao, et al., 2006). Yet no such interaction is seen with application of rhapoE2 and rhapoE4 isoforms despite enriching for NMDAR subunits by immunoprecipitation of NR2A or NR2B to increase signal-to-noise ratios for quantitative western blot analysis. There is a trend towards decreased tyrosine phosphorylation in the presence of apoE isoforms as compared to control levels (figure 7). Although not statistically significant, this trend could be indicative of reduced phosphorylation of NMDARs and may contribute, at least in part, to the observed decreases in NMDAR-mediated field potentials. However, this trend towards reduced tyrosine phosphorylation is seen in the presence of all three apoE isoforms, including apoE3, which does not have an effect on NMDAR-mediated field potentials with acute application.

Nevertheless, site-specific loss of phosphorylation is likely to have a differential effect on NMDAR function. Importantly, quantification of phosphorylation was performed using a pan-antibody for phosphorylated tyrosine and specific sites of tyrosine phosphorylation were not identified. Both NR2A and NR2B contain a number of specific tyrosine phosphorylation sites that are acted upon by specific protein kinases (Cheung & Gurd, 2001; Gardoni, et al., 2001; Liao, et al., 2001; Nakazawa, et al., 2001; Omkumar, et al., 1996; Yang & Leonard, 2001; Zheng, et al., 1998). While the functional significance of many of these sites remains inconclusive, alterations in phosphorylation at one or several sites could potentially alter NMDA receptor function in a way that impacts synaptic plasticity. NMDA receptor activity can be upregulated through phosphorylation

by SFKs (for review see (Ali & Salter, 2001)). This enhancement can be blocked by mutating specific tyrosine residues in NR2A (Y1105, Y1267, Y1387) to phenylalanine (Zheng, et al., 1998). Paradoxically, the principal site of fyn phosphorylation in NR2B, Y1472, does not correspond to the src sites in NR2A (Nakazawa, et al., 2001). This phosphorylation of this site, however, does increase following LTP induction in hippocampal slices and after transient global ischemia (Nakazawa, et al., 2001). The equivalent Y1472 site in NR2A surrounded by a clathrin adapter-2 (AP2)-binding domain; this can mediate NMDA-receptor internalization when not masked by binding to the PDZ domain of PSD-95 (Roche, et al., 2001). Therefore increase in tyrosine phosphorylation of this site may regulate receptor turnover. A decrease in NR2A tyrosine phosphorylation in the presence of rhapoE could thus alter NMDAR function or surface expression, and either possibility could manifest as a reduction in NMDAR-mediated field potentials.

Furthermore, the cytoplasmic tails of NMDA receptors also are subject to phosphorylation by other kinases, such as CDK5. CDK5 is found localized with NMDA receptors at glutamatergic postsynaptic sites (Li, et al., 2001). Inhibition of CDK5 reduces current through the NMDA receptor and blocks LTP induction in hippocampal slices (Li, et al., 2001), illustrating the importance of non-tyrosine phosphorylation in the regulation of NMDA receptor function. Hyper-phosphorylation of these non-tyrosine residues could potentially mask immunoreactivity of other sites. Such an interaction could account for the differing results seen with immunoprecipitation of NR2A and NR2B versus total CA1. Thus, in this context, slight changes in NMDAR total tyrosine

phosphorylation can have unknown changes in NMDAR function, such as either promoting or inhibiting LTP induction.

Other than altered phosphorylation, there can be actual physical changes that would result in reduced NMDAR function. This is a distinct possibility in light of recent studies showing that apoER2 is physically associated with NMDA receptors by PSD-95 (Beffert, et al., 2005; Hoe, Pocivavsek, Chakraborty, et al., 2006). Increased receptor binding and endocytosis in the presence of apoE2 or delayed retroendocytosis in the presence of apoE4 could therefore alter the distribution of NMDA receptors on the cell membrane. Furthermore, apoER2 internalization could also alter NMDAR function by disruption of signal transduction pathways known to modulate NMDA receptors (Beffert, et al., 2002; Petit-Turcotte, et al., 2005; S. Qiu, Zhao, et al., 2006; Weeber, et al., 2002). The resulting effect would be an isoform-specific decrease in NMDAR-mediated field potentials in the presence of apoE2 and apoE4 but not apoE3.

Acute application of apoE isoform does not significantly alter activation of ERK1/2 or JNK1/2 (figure 8). These signal transduction pathways are key players in synaptic plasticity, and were previously shown to be modulated by apoE application in neuronal cultures (Hoe, et al., 2005). The absence of changes in ERK1/2 and JNK1/2 with acute apoE application in this study is interesting in light of the fact that reelin application does not change ERK1/2 activation (unpublished observation). The ability for apoE to bind to all lipoprotein receptors, in particular apoER2 and VLDLR, raises the possibility that acute apoE application can significantly modulate normal reelin signaling. ApoE2 and apoE4 may induce greater receptor internalization or slower turnover than

apoE3. This is supported by findings showing reduced retroendocytosis of the apoER2 receptor in the presence of apoE4 (J. Herz, personal communication). While apoE2 is deficient in binding LDLR, its affinity for apoER2 is unknown. The finding of increased apoE2 levels by other groups (Riddell, et al., 2008; Sullivan, et al., 2004), however, may increase the probability of receptor binding by this isoform thereby decreasing receptor availability for reelin. ApoE3, in contrast, displays neither of these traits.

Simultaneously, apoE2 and apoE4 could have divergent effects on reelin processing and metabolism. Preliminary results suggest an increase in active reelin fragments in the presence of apoE4, and a decrease in the presence of apoE2 (data not shown). In addition, apoE4 expression occludes reelin-induced enhancement of LTP (J. Herz, personal communication). Thus, apoE4 application may increase the signal-to-noise ratio of reelin signaling, priming the synapses for increased response to stimulation which manifests as an increase in LTP induction (see figure 9).

Implications of chronic apoE expression

The creation of the apoE TR model allows for expression of specific apoE isoforms that is "chronic" and unchanging throughout development and postnatal life. Chronic apoE expression in apoE TR mice contrasts many of the findings seen with acute apoE exposure. The isoform-specific alterations in LTP induction require NMDA receptor function, but determining the mechanism of these NMDAR-dependent changes proved difficult. These technical difficulties arose in part due to the potential confounds and caveats that would accompany investigation of the entire hippocampus versus area

CA1. For example, attempts to immunoprecipitate NR2A or NR2B from apoE TR CA1 samples resulted in a high noise to signal ratio ultimately leading to widely varying levels of total subunits detected (figure 14). The magnitude of these changes combined with a lack of change in NR1 levels brought the validity of these results into questions. A parallel study of total CA1 extracts without immunoprecipitation failed to replicate the isoform-specific changes in subunit or phosphorylation levels. This lack of confirmation indicates that the current immunoprecipitation protocol does not adequately control for the differential treatment of NMDA receptors with chronic apoE isoform exposure. The observed hyper-phosphorylation may be indicative of other apoE-dependent effects, such as changes in receptor localization that will be determined in future studies.

Although the effect of apoE expression on NMDAR function is unclear, the effects on signal transduction are much more apparent. Both ERK1/2 and JNK1/2 activation are significantly increased in apoE4 TR animals versus apoE2 TR and apoE3 TR. It is likely that the changes to synaptic plasticity depend heavily on these changes in neuronal signal transduction. JNK interacting protein (JIP) interacts with the amino acids encoded by exon 19 of apoER2. This sequence is also required for interaction with PSD-95, reelin-induced increases in LTP, and reelin-induced increases in NMDA receptor subunit phosphorylation and potentials (Beffert, et al., 2005). Likewise, ERK1/2 function is also necessary for synaptic plasticity. NMDAR-dependent LTP in CA1 is associated with an increase in active ERK2 (English & Sweatt, 1996), and this type of LTP is blocked by inhibition of MAP kinase kinase (MEK), the sole activator of ERK2 (English & Sweatt, 1997). Isoform-dependent activation of these pathways is therefore implicated

in alterations in LTP induction. However, the results presented here cannot eliminate alterations to NMDA receptor function or indirect effects on reelin signaling as contributors to the apoE isoform-dependent changes in LTP induction. As illustrated by figure 16, it is more likely that the cooperative effect of changes in signal transduction and potential alterations to NMDAR phosphorylation are together combining to alter synaptic plasticity in the chronic expression state.

Alterations in apoE binding may also change the ability of lipoprotein receptors to bind other ligands, such as reelin. The previous reports of reduced levels of apoE4 in the brain (Ramaswamy, et al., 2005; Riddell, et al., 2008) could correlate with increases in the number of receptors available for reelin binding. Increased reelin levels, and presumably increased binding to receptors, could initiate signal transduction cascades culminating in enhanced LTP induction much like that seen with apoE4 expression. Stability of apoE conversely mirrors LTP induction, with the least stable apoE4 inducing the greatest LTP induction and the most stable apoE2 the least. Combined with the finding of reduced spatial memory, this suggests that the increase in LTP with apoE4 may be a pathological release of inhibition. The neuroprotective effects of apoE2 may stem from an ability to suppress excess stimulation. This is complemented by finding that while modest increases in reelin enhance learning, excess reelin actually inhibits learning and memory.

The findings of this study are summarized in table 3. While contributing to our knowledge about the effects of apoE isoform, the results presented here also raise another interesting question: Why is there a mechanistic difference between acute and chronic

Table 3: Summary of electrophysiology and biochemistry results: comparison between acute (rhapoE treatment) and chronic (apoE TR) conditions

	Acute (rhapoE)	**Chronic (apoE TR)**
5x TBS LTP	E4 > E2	E4 > E2
200 Hz LTP	n.d.	n.s.
NMDAR-mediated field potentials	E2, E4 < control	n.d.
pNR2A/NR2A (IP of NR2A)	n.s. (trend towards reduced pNR2A in E4)	E4> E2, E3
pNR2B/NR2B (IP of NR2B)	n.s.	E4> E2, E3
NR2A/NR1 (total CA1)	n.d.	n.s.
NR2B/NR1 (total CA1)	n.d.	n.s.
pTyr (total CA1)	n.d.	n.s.
pERK/ERK	n.s.	E4> E2, E3
pJNK/JNK	n.s.	E4 > E2, E3 WT> E0

n.s. - not significant
n.d. - not determined

apoE isoform treatments? It is possible that the mechanistic differences simply stem from the duration of apoE exposure, and that extended exposure to recombinant apoE isoforms would eventually mimic exactly the physiologic and biochemical changes seen in the apoE TR mice. However, the two treatments, with their apparent divergent mechanisms, both result in the same apoE isoform-dependent changes in LTP. While this finding should not be discounted, the results of this study suggest that biophysical differences between recombinant and endogenous apoE may separate these two models. These biophysical differences, and their implications, will be discussed in the next section.

Molecular implications of apoE isoform variation

Deciphering the biophysical properties of apoE isoforms is beyond the scope of this study and is currently under investigation by many other research laboratories. However, the biophysical properties of individual apoE isoforms support my hypothesis that apoE can act as an isoform-specific signaling molecule. Each isoform has a specific affinity for particular lipoproteins and receptors. This in turn alters the extent to which apoE induces receptor endocytosis and intracellular signaling. Furthermore, variation in the level of available apoE due to variation in stability or expression can also alter the amount of receptor binding and signaling by apoE. In the apoE TR animals, these interactions are presumably preserved and thus the isoform-specific changes in synaptic plasticity, learning and memory are a manifestation of these biophysical properties. In contrast, the rhapoE isoforms are applied without lipids. The disconnect between the

actions of these rhapoE isoforms and the results obtained with apoE TR animals may reflect the particular processes that are dependent upon proper binding of apoE/lipoprotein complexes to receptors. This also emphasizes the importance of using naturally lipidated apoE particles to determine the mechanism of apoE isoform-dependent alterations in synaptic plasticity.

There may be additional explanations for the disconnect in the data collected between the acute and chronic experiments. In the apoE TR animals, apoE isoforms are present throughout life and presumably exerting any isoform-specific differences on signaling, synaptic plasticity, and behavior continuously. Thus the measured differences between isoforms are a reflection of both signaling changes in the adult animal as well as any compensatory changes necessary to maintain homeostasis. Similarly, the absence of apoE can also induce signaling or other compensatory changes. However, with the exception of JNK activation, there were no significant differences in synaptic plasticity or signaling measure in apoE-deficient mice as compared to wild-type mice in this study. Application of rhapoE isoforms is sufficient to mimic changes in LTP induction but not changes in ERK1/2 activation. There are at least three possible explanations for this: 1) rhapoE is not inducing the same signaling pathways as endogenous apoE due to the absence of physiologically relevant lipidation, 2) rhapoE is inducing signaling but there are additional mechanisms at work that reverse these changes prior to the time points measured in this study, or 3) these alterations are compensatory changes in the apoE TR mice that will not be seen with acute application of rhapoE.

Support for the first possibility has been raised in both the introduction and in this section; apoE isoform affects lipoprotein affinity, and the state of lipid binding to apoE has implications for receptor interactions. The second possibility is strengthened by the different time courses used in this study. In the synaptic plasticity experiments, rhapoE is applied to slices for five minutes and remains in perfusion for 20 minutes prior to LTP induction and 20 minutes after. For the biochemistry experiments, rhapoE is in perfusion for the 40 minutes prior to flash freezing on dry ice to terminate cellular processes. Thus, there is a 20 minute period in which any changes in signaling that lead to the observed alterations in synaptic plasticity could be reversed.

Treatment of primary neurons with apoE activates ERK1/2 in a time dependent fashion. Levels of pERK are equal to control after 30 minutes, peak at 2 hours, and are near control levels again by 6 hours (Hoe, et al., 2005). While the time course will be different in hippocampal slices, it is feasible that similar time-dependent effects are occurring in this study. Application of a bolus of rhapoE could quickly induce changes in ERK1/2 activation, NMDAR phosphorylation, or other cellular pathways that peak at or near 20 minutes of exposure. Subsequently, mechanisms to reverse these changes, such as activation of protein phosphatases or receptor desensitization, return the system to levels that are indistinguishable from control in the assays used in this study. An in-depth analysis of the time course of rhapoE-induced activation of signaling pathways will help validate this possibility.

In the apoE TR mice, chronic exposure to apoE isoforms has resulted in homeostatic changes that are not seen with acute exposure. For instance, apoE isoform

expression could change the rate of lipoprotein receptor turnover, surface level expression of lipoprotein receptors, or NMDA receptor desensitization. In this study, chronic apoE4 expression results in a significant increase in ERK1/2 activation. This could be the result of compensatory changes in signaling that are necessary to maintain homeostasis. In addition, this elevated ERK1/2 activation could result in downstream inhibition to the targets of the MAPK cascade, such as cAMP response element binding (CREB). Inhibition of CREB and downstream gene transcription processes could result in learning and memory deficiencies in the apoE4 TR mice. Neither of these effects would occur with acute application of apoE. Therefore, the failure of acute apoE application to replicate all of the changes in signal transduction seen in the apoE TR mice may stem from the effects of chronic apoE isoform expression rather than the properties of rhapoE itself.

Implications for learning and memory

One great utility of the apoE TR mouse is the ability for this model to undergo behavioral testing to determine specific effects of naturally lipidated, chronic apoE exposure on learning and memory. While there is no overall change in general locomotion, this study confirmed deficits in spatial memory in apoE4 animals, expanding this to include male animals as well as the previously studied female animals (Grootendorst, et al., 2005). In addition to this spatial memory deficit, there is a significant difference in associative learning ability between apoE2 and apoE4 TR mice.

A comparison of the findings of this study with the three other major studies focusing on the apoE TR mice (Bour, et al., 2008; Grootendorst, et al., 2005; Villasana, et al., 2006) is presented in table 4. As evidenced by this table, small differences in experimental design can alter the behavioral profile of identical strains of animals. This does not take into consideration other immeasurable influences on behavior, such as housing conditions or experimenter handling, or data interpretation, such as the sensitivity limits of a particular behavioral apparatus.

Likewise, the effects of the absence of apoE on learning and memory are somewhat controversial. In several studies, spatial learning is impaired in the absence of apoE (Champagne, et al., 2002; Grootendorst, et al., 2001; Krzywkowski, et al., 1999; Masliah, et al., 1997; Oitzl, et al., 1997; Veinbergs, et al., 1999). These differences may be attributable to procedural differences in training protocols, for instance massed training sessions instead of spaced (Oitzl, et al., 1997), or sex or age differences in the mice used in those studies (Masliah, et al., 1997; Veinbergs, et al., 1999) versus the young adult male mice in this study. In others studies, spatial memory is equivalent between apoE-deficient and murine apoE-expressing animals (Anderson, et al., 1998; Anderson & Higgins, 1997; Grootendorst, et al., 2005; Raber, et al., 1998; Raber, et al., 2000).

In this study, apoE-deficient animals are behaviorally indistinguishable from wild-type and apoE3 TR animals in many tasks. One possible interpretation of this finding is that murine apoE and apoE3 do not have an effect on these behaviors, in essence doing “nothing.” While this is a possibility, other studies show that lack of apoE does in fact have deleterious effects on learning and memory. Therefore, it is more likely that the

Table 4: Summary of behavioral studies of apoE TR mice: comparison between current and previous studies

	Current Study	Grootendorst et al., 2005	Bour et al., 2008	Villasana et al., 2006
genotypes	ApoE2, apoE3, apoE4 TR C57BL/6J apoE deficient	apoE3, apoE4 TR C57BL/6J apoE deficient	apoE3, apoE4 TR C57BL/6J apoE deficient	apoE2, apoE3, apoE4 TR
origin of mice	TR: Taconic (P. Sullivan) WT: Jax E0: Jax	TR: P. Sullivan WT: Charles River E0: Charles River	TR: P. Sullivan WT: Charles River E0 Charles River	TR: P. Sullivan
age	3-5 months	4-5 months	15 months	5 months (sham cranial irradiation at 2 months)
sex	male	male and female	male and female	male and female
open field - distance traveled	n.s.	E3 > E4, WT, E0	E3 > E4, WT, E0	E3 > E4 female > male
rotorod - average latency to fall	E3 < E2, E4	n.d.	n.d.	E3 < E2, E4 (sex matched)
acoustic startle	E2 < E3, E4	n.d.	n.d.	n.d.
PPI	n.s.	n.d.	n.d.	n.d.
shock threshold	WT > E0 and E3 < E2 at low intensities,	n.s.	no genotype effect	n.d.
object recognition - protocol	Novelty test: - 2 objects - 4 trials: 5 min with objects (x3) 5 min with 1 novel and 1 familiar object - 5 min ITI	Spatial test: - 5 objects - 3 trials: 5 min no objects, 15 min with objects, 15 min with 2 objects, relocated - 5 min ITI	Spatial test: - 5 objects - 3 trials: 5 min no objects, 15 min with objects, 15 min with 2 objects, relocated - 5 min ITI	Spatial and novelty test: - 3 objects - 5 trials 10 min with objects (x3) 10 min with 1 object moved 10 min with 1 familar object replaced with novel - 3 min ITI

n.s. - not significant   n.d. - not determined

Table 4 (continued): Summary of behavioral studies of apoE TR mice: comparison between current and previous studies

	Current Study	**Grootendorst et al., 2005**	**Bour et al., 2008**	**Villasana et al., 2006**
object recognition - results	All groups show preference for novel object	Performance of female E4 reduced versus female E3 and female E0	Performance of female E3 reduced (versus chance)	All groups show preference for object in new location and novel object
fear conditioning - training	n.s. between groups	n.d.	n.d.	n.d.
fear conditioning - context (percent freezing)	1 hr - n.s. 24 hrs - significant difference between E2 and E4	n.d.	n.d.	n.d.
water maze training -protocol	- 4 trials/day for 8 consecutive days -ITI ~1 hr - probe trial at **1 and 24 hrs** after last training	- 4 trials/day for 4 consecutive days - ITI 5-10 min - probe trial at **24 hrs** after last training	- 4 trials/day for 4 consecutive days - ITI 5-10 min - probe trial at **24 hrs** after last training	- 3 trials/session, 2 sessions/day for 3 days - ITI 10-15 min, ISI 3.5 hrs - probe trial **1 hour** after last training
water maze training -result	All animals improve performance (decrease mean swim distance) over training days	Female E3 do not improve mean swim distance over training days	All animals improve performance (decrease mean swim distance) over training days	All animals improve performance (decrease mean swim distance) over training days
water maze probe trial	mean time in target quadrant: 1 hr - significantly greater than other quadrants for all groups 24 hr - significantly greater than other quadrants for WT, E0. significant vs opposite for E2, E0. n.s. for E4	mean time in target quadrant: All significantly greater than chance EXCEPT E4	mean time in target quadrant: significantly greater than chance for E3, WT, E4 males, E0 females (not E0 males, E4 females)	mean time in target quadrant: significantly greater than time in other quadrants for all groups

n.s. - not significant   n.d. - not determined

experimental conditions or design of individual tests in this study are unable to distinguish the subtle consequences due to the lack of apoE. Interestingly, these tests are able to identify changes in learning and memory due to the presence of apoE2 or apoE4. As no corresponding alterations are seen in apoE3 TR animals, this indicates that alterations seen in apoE2 TR and apoE4 TR animals are specifically related to the presence of these isoforms.

The findings presented here and the ongoing study into the biochemistry of apoE signaling will help elucidate the mechanism of apoE isoform-dependent modulation of cognitive function. Despite this progress, it is imperative to note that it is exceedingly difficult to directly associate learning and memory with neuronal physiology. While there are multiple instances where increases in LTP correspond to increases in learning and memory performance, there are also circumstances of inverse correlation or no relationship at all between behavior and synaptic plasticity. For example, animals lacking Fmr2, disruptions of which lead to FRAXE mental retardation syndrome in humans, display enhanced LTP but are severely impaired in long-term contextual associative memory (Gu, et al., 2002). Similarly, mutation of PSD-95 increases LTP induction but significantly reduces spatial learning and memory (Migaud, et al., 1998).

In this study, increases in synaptic plasticity in the apoE4 TR animals correlated with both an increase in ERK1/2 activation and a decrease in spatial memory retention. Hippocampal ERK activation is necessary for spatial memory formation. Administration of the MEK inhibitor SL327 in mice selectively impairs performance on the hidden platform test (Selcher, et al., 1999). Likewise ERK activation is seen in the CA1/CA2

subfield hippocampus following watermaze training (Blum, et al., 1999). Yet in the current study, both decreased spatial memory and increased ERK activation is seen in the apoE4 TR animals. The possibility exists that the observed increase in basal ERK activation actually interferes with the formation of new long-term memories. If hippocampal ERK activation and synaptic plasticity is constantly elevated prior to learning and memory, then this could interfere with the induction of changes necessary for the encoding of a persistent memory trace in the hippocampus.

A key consideration in the interpretation of these findings is the idea of occlusion. If apoE4 expression increases LTP and activates signaling pathways in the basal state, then further increases in LTP may not be possible. Thus learning deficits should arise due to a loss of capacity for hippocampus synaptic plasticity. Saturating hippocampal LTP does impair spatial memory (Castro, et al., 1989; McNaughton, et al., 1986). Specifically, animals that receive saturating LTP stimulation to the perforant path do not spend significantly more time in the target quadrant during a probe trial following water maze training (Moser, et al., 1998). In contrast, animals receiving non-saturating LTP stimulation, low frequency stimulation, or no stimulation are not impaired in spatial learning (Moser, et al., 1998). Thus, apoE4 expression supports the point that more is not always better. The observed changes in LTP and signal transduction in these animals may indicate a reduction in the capacity for further alterations in synaptic plasticity.

Due to its negative association with AD risk, cardiovascular disease risk, and prognosis following traumatic brain injury, apoE4 has been portrayed as a biological villain in much of the literature. Interestingly, the apoE4 allele is carried at different

frequencies among world populations (Corbo & Scacchi, 1999), and the preservation of this allele despite disease risk might indicate an advantageous ancestral role for this allele. Several human studies have indicated that apoE4 bestows beneficial properties early in life but escalate deficiencies with age. ApoE4 has been associated with increased intelligence in young women (Mondadori, et al., 2007), but a more rapid cognitive decline in older women (Mortensen & Hogh, 2001). Young apoE4 carriers also have differences in cerebral blood flow as measured by BOLD fMRI, most notably an increase in hippocampus activation during memory encoding (Filippini, et al., 2009). A longitudinal study of individuals from age 11 to 70 did not find a correlation between IQ and apoE isoform expression in children, but childhood IQ and apoE did interact to affect processing speed and nonverbal cognition in old age (Luciano, et al., 2009). Likewise, the increased LTP seen in young adult apoE4 TR may be a manifestation of changes in neuronal processes that, with age, have a progressive negative impact on cognitive function.

Experimental limitations and considerations

The findings of this study stress the importance of using physiologically relevant forms of apoE to study isoform-dependent effects on synaptic plasticity. The recombinant apoE isoforms used in this study were non-lipidated. Various forms of lipidated and non-lipidated apoE have been used somewhat interchangeably in the literature, from apoE peptides and recombinant apoE, to artificially lipidated apoE, to CSF-derived apoE/lipoprotein particles (Bellosta, et al., 1995; Fagan, et al., 1999; Hoe, et

al., 2005; Hoe, Pocivavsek, Dai, et al., 2006; LaDu, et al., 1995). This lipidation state, however, has significant consequences for the structure and domain interaction of apoE. Domain interaction of apoE4 depends on lipid type (Hatters, et al., 2005). Additionally, apoE must be bound to lipids for full, high-affinity binding to receptors (Hatters, et al., 2006) and removal of Aβ (Hirsch-Reinshagen, et al., 2005; Jiang, et al., 2008; Wahrle, et al., 2005; Wahrle, et al., 2004). This could have unforeseen consequences on studies using recombinant or non-lipidated forms of apoE. To minimize these confounding variables, future synaptic plasticity studies should focus on forms that preserve physiological lipidation, such as glial-derived apoE (Manelli, et al., 2007) or the apoE TR animals.

The apoE TR animals, however, are not without their own limitations. While the endogenous murine apoE promotor is preserved, the remainder of the apoE gene is human. The gene product therefore is, in essence, foreign. As murine apoE behaves like human apoE3, the conformational differences of apoE2 or apoE4 may have consequences on expression, transport, or reuptake. Therefore, this study operates under the assumption that human apoE isoforms are able to effectively interact with the murine system. Fortunately, the apoE receptors involved in transducing apoE-dependent signals across the membrane are highly conserved across species, and in particular between mammals with 90-97% sequence similarity depending on the specific apoE receptor (Herz & Bock, 2002). Thus, when presented in a physiologically relevant lipidation state, human apoE isoforms should be able to interact with murine apoE receptors in a way that reflects their

differential binding abilities in the human. However, further study will be necessary to confirm this assumption.

The apoE TR animals used in this study were homozygous for each particular apoE allele. In using these animals, we are attempting to model chronic apoE expression that can be related human conditions. In reality, however, the likelihood of an individual possessing two ε2 or two ε4 is quite low (Corbo & Scacchi, 1999; Siest, et al., 1995). This limits the ability to directly translate the findings of this study to the human brain as there may be interactions between different apoE isoforms that have additional effects on synaptic plasticity.

Summary

In total, the results of this study demonstrate a specific, isoform-dependent role for apoE in the modulation of synaptic plasticity and cognitive function. ApoE4 increases LTP induced by theta burst stimulation in area CA1 of the hippocampus; the magnitude of this LTP induction is significantly greater than that seen in the presence of apoE2. This change in LTP induction is accompanied by increased ERK1/2 and JNK1/2 phosphorylation when apoE4 is chronically expressed in the apoE TR mouse but not when acutely applied. Acute apoE2 and apoE4 application significantly decreases NMDAR-mediated field potentials but this is not accompanied by a significant decrease in tyrosine phosphorylation of NR2A or NR2B. The effects of chronic apoE expression on NMDAR function are less clear. While NMDA receptors are required for the isoform-specific effects on LTP induction, subunit expression and phosphorylation levels were

equivalent in total CA1 samples. Attempts to immunoprecipitate NR2A and NR2B revealed increased tyrosine phosphorylation and decreased subunit detection in the presence of apoE4.

As illustrated by figures 9 and 16, apoE may induce isoform-specific changes in synaptic plasticity through alterations in signaling cascades, namely ERK1/2 and JNK1/2, and NMDAR function. While having the same final effect on theta burst-induced CA1 LTP, acute apoE exposure and chronic apoE expression may be divergent in mechanism. Chronic apoE expression, specifically apoE4, alters signaling cascades known to be involved in synaptic plasticity, and likely alters NMDAR function although this remains unconfirmed. Acute apoE exposure, however, does not alter these signal transduction cascades but does reduce NMDAR-mediated field potentials. Further experimentation will be required to determine if these differences are attributable to the lack of physiologically relevant lipidation of the acutely applied apoE, the time course of apoE application, adaptation due to the chronic expression of apoE isoform, or a combination of these factors.

The apoE isoform-specific modulation of synaptic plasticity is complemented by alterations in learning and memory performance in the apoE TR mice. This study builds upon previous research, confirming a spatial memory deficit in apoE4 TR mice. Furthermore, apoE2 TR mice also have impaired spatial memory, and there is a significant difference in associative memory performance between apoE2 and apoE4 TR animals. These changes are seen without any alterations to general locomotion, motor learning, or working memory. While direct correlations can not be made between LTP

and learning and memory behavior, both parts of this study show more dramatic apoE isoform-induced changes in the presence of apoE2 or apoE4 versus apoE3. Overall, this study demonstrates that apoE isoform specifically acts to modulate neuronal signaling in the adult hippocampus resulting in alterations to both synaptic plasticity and learning and memory and establishes a baseline for future studies into the role of apoE isoforms in the human CNS.

Future directions

The findings presented here bring valuable insight to the growing apoE field. The identification of baseline levels of synaptic plasticity and learning and memory behavior in apoE TR mice creates a foundation for future research. The clear caveat to these studies is that the apoE TR animals are expressing human apoE but possess receptors designed for the binding and uptake of mouse apoE. However, the family of apoE receptors likely involved in transducing these signals across the membrane are highly conserved across species, and in particular between mammals with 90-97% sequence similarity depending on the specific apoE receptor (Herz & Bock, 2002). The apoE TR mice are therefore valuable for future studies combining apoE isoform expression with other AD models or alterations in lipoprotein receptor signaling.

Studies combining apoE expression with other manipulations have already begun. In apoE TR mice crossed with a mouse model of AD, the PDAPP mouse, expression of apoE4 dramatically increases brain Aβ levels. This is accompanied by isoform-specific changes in brain apoE, E4<E3<<E2, but no change in apoE mRNA by isoform (Bales, et

al., 2009). Estradiol treatment in aged apoE TR mice to mimic hormone replacement therapy selectively increased perforant path LTP in apoE4 TR versus apoE3 TR (Yun, et al., 2007) but these aged apoE4 TR animals did not show the same basal reduction in perforant path LTP previously reported in young animals (Trommer, et al., 2004). Induction of experimental autoimmune encephalomyelitis, a model of multiple sclerosis, in apoE4 knock-in mice induces an early spatial memory decline and a decline in choline acetyltransferase in the hippocampus as compared to wild-type animals (Tu, et al., 2009). With the results presented in this study, the interpretation of these and future results can take basal changes in synaptic plasticity and learning and memory due to apoE4 expression into consideration. Furthermore, the studies listed above underscore the ability of non-murine apoE isoforms to elicit physiological changes related to human disease states.

Another interesting finding of my research is the effect of apoE isoform on NMDA receptors. A full characterization of this effect first requires validation of the experimental procedures, especially immunoprecipitation, used to isolate and analyze NMDA receptor subunits in this study. This assay can then be used to measure changes in NMDAR subunit composition or phosphorylation in the presence of apoE isoforms, and may also reveal alterations to subcellular localization or surface distribution. The present studies revealed changes changes in pan-phosphotyrosine levels; however identification of specific tyrosine phosphorylated residues will help further refine the mechanism of apoE activity in the hippocampus. Future studies may also determine that activation of other signaling pathways is involved in phosphorylation of NMDA receptors

at other residues. For example, there was no investigation of serine or threonine phosphorylation of NMDARs that can also influence calcium conductance. These types of biochemical investigations would be technically difficult and time consuming, but may shed light on both apoE-dependent changes in synaptic function as well as biophysical properties of NMDA receptor function

Insight in to the mechanism of the biochemical effects of apoE isoforms can also be gathered from additional plasticity studies. For instance, apoE isoform presumably alters synaptic plasticity through lipoprotein receptors but does this then occlude reelin-dependent changes in plasticity? Likewise, we now know that apoE isoforms have different effects on perforant path LTP versus CA1 LTP; does this correlate with any differences in the activity of reelin between these two areas? Reelin enhances LTP in area CA1 but the effect of reelin application of perforant path LTP is unknown. This simple experiment may strengthen the link between the integration of reelin signaling and apoE signaling in the hippocampus. Along the lines of further plasticity study is the investigation of isoform specific changes in other forms of synaptic plasticity, such as LTD or chemically induced LTP (i.e. phorbol-ester induced synaptic facilitation). These types of studies will help define changes induced by both acute and chronic apoE application and can be useful in understanding the potential mechanisms underlying cognitive deficits in the apoE TR mice

Additional studies utilizing whole cell electrophysiology could isolate the particular modulation to ion channels that contribute to changes in synaptic plasticity. These studies in particular would benefit from the isolation and use of lipidated apoE

isoforms. If these particles mimic the physiological state, application during whole cell recording would allow for the investigation of the time course of apoE signaling. In addition, this would be the ideal experimental set-up for the investigation of the pathways of apoE signaling as inhibitors of particular kinases, such as SFKs or PI3K, could be either bath applied or delivered intracellularly via the recording pipette.

Further insight into the mechanism of apoE isoform-dependent changes on synaptic plasticity could also be garnered by breeding strategies that incorporate apoE TR mice in combination with one of the many lipoprotein-deficient transgenic mice or by utilizing molecules that block ligand binding to specific lipoprotein receptors. This would confirm that apoE isoforms are acting through lipoprotein receptors as well as indicate which receptors are expressly involved in mediating apoE-specific effects. The identification of the specific lipoprotein receptors involved in apoE isoform-dependent changes to synaptic plasticity will also hint at the role of reelin in this process. This will be further clarified by continuation of the study of the role of apoE isoform in reelin metabolism. Recent work in the Weeber lab indicates that apoE4 increases metabolism of reelin to produce the active fragment. In contrast apoE2 reduces metabolism and reduces the amount of active fragment. The differential effect of apoE isoform on reelin may support the different effects of apoE isoforms on plasticity and learning and memory.

The ultimate goal of all of the research into isoform-specific actions of apoE is to understand how these changes culminate into altered AD risk. While the apoE TR animals do not develop AD-like pathologies, they nonetheless could be used as an animal model for the testing of therapeutic interventions. In the apoE4 TR mouse, for example,

the success of a therapeutic could be measured by its ability to return the electrophysiological, biochemical, and behavioral profile to that which resembles the apoE3 TR. The interaction between apoE and lipid metabolism makes the investigation of dietary interactions especially interesting. For instance, there is currently controversy in the literature about the effectiveness of omega-3 fatty acids, specifically docosahexenoic acid (DHA), in the progression of AD symptoms and pathologies (Devore, et al., 2009; Hashimoto, et al., 2008; Hooijmans, et al., 2009). Lim *et al.* reported a decrease in Aβ levels with dietary DHA supplements in an aged animal model of AD (Tg2576 mice) (Lim, et al., 2005). Two recent studies in humans, however, suggest restraint should be used when recommending DHA supplements to AD patients. Quinn *et al*. found that DHA treatment did not slow mental decline in AD patients. However, there appears to be an effect of apoE isoform, as non-ε4 carriers have a slower rate of decline than those with the ε4 allele (J. F. Quinn, et al., 2009). In addition, DHA supplements improve memory function in healthy adults with age related cognitive decline (Yurko-Mauro, et al., 2009). The interaction between DHA and apoE4 could be further investigated in the the apoE TR which may ultimately lead to a refinement of dietary therapeutic recommendations for AD patients.

Conclusion

The identification of apoE isoform expression influencing the risk of developing AD was a seminal finding and refocused much attention of the AD research community on the role of specific apoE isoforms in AD pathology. Incredibly, there has been little

attention given to the potential role of these isoforms as signaling molecules in the CNS or their role in synaptic function and memory formation. Until these potential roles have been identified a full appreciation of how isoform expression integrates into AD symptomology, etiology and pathology will never be realized. The studies described within this dissertation represent the important first-step in addressing the deficiencies in this area of AD research. Here I have performed a broad-based characterization of basic behavioral and physiologic changes in a mouse model expressing human proteins. In addition, restricted biochemical and electrophysiological experimentation was included to provide an insight on possible mechanisms underlying any isoform specific changes in learning and memory.

The studies within this dissertation show for the first time that there is an isoform-dependent modulation of LTP induction in area CA1 of the hippocampus that appears to be dependent on NMDA receptor activation. In addition, expression of specific isoforms can affect at least two signal transduction pathways that are well known for their involvement in synaptic plasticity and memory formation. Moreover, these biochemical and physiologic changes may underlie perceptible differences in learning and memory in adult male mice. Taken together, the novel results presented here firmly establish apoE isoforms as a modulator of both synaptic plasticity and learning and memory, and provide a foundation for future apoE research that can be integrated into forthcoming models of Alzheimer's disease.

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