MICROGLIAL LRP1 MODULATES JNK ACTIVATION: A SIGNALING CASCADE THAT ALSO REGULATES APOLIPOPROTEIN E LEVELS

A Thesis
submitted to the Faculty of the
Graduate School of Arts and Sciences
of Georgetown University
in partial fulfillment of the requirements for the
degree of
Doctor of Philosophy
in Neuroscience

By

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Washington, DC
March 26, 2009
Apolipoprotein E (apoE) has been implicated in modulating the central nervous system (CNS) inflammatory response. However, the molecular mechanisms involved in the apoE-dependent immunomodulation are poorly understood. We hypothesize that apoE alters the CNS inflammatory response by signaling via low-density lipoprotein (LDL) receptor family members in glia. To address this hypothesis, we used a small bioactive peptide formed from the receptor-binding domain of apoE, apoE peptide (EP), to study LDL receptor family signaling in microglia. To model glial activation, we treated primary mouse microglia and the microglial cell line BV2 with lipopolysaccharide (LPS) and studied two inflammatory responses: an increase in nitric oxide production (NO) and a decrease in apoE production. We found that treatment of microglia with EP attenuated LPS-induced NO accumulation and apoE reduction via LDL receptor family members. We found that EP decreased JNK activation and thereby suppressed the LPS-induced JNK activation, which proved essential to increase NO and decrease apoE production. Next, we investigated whether activation of low-density lipoprotein receptor-related protein 1 (LRP1), a member of the LDL receptor family, mediated the anti-inflammatory effects of EP. To address this hypothesis, we studied LPS-induced
inflammation and EP-induced signaling in primary microglia that were LRP1-deficient. In these studies, EP did not attenuate LPS-induced NO accumulation and JNK activation in LRP1-deficient microglia. Thus, we concluded that activation of LRP1 is essential for EP-induced anti-inflammatory properties in microglia. Lastly, we investigated the impact of JNK inhibition on apoE production in the brain. For these studies, we used two JNK inhibitors, JNK inhibitor I (L)-form (L-JNK1) and SP600125. We studied changes to apoE levels in primary glia treated with JNK inhibitors and mice injected with JNK inhibitor. Using APOE promoter luciferase reporter constructs, we found an increase in APOE promoter activity when JNK was inhibited. Our studies showed that APOE transcription and apoE protein levels are increased with JNK inhibition. We suggest that JNK inhibitors may prove useful by increasing apoE and its protective anti-inflammatory properties.
ACKNOWLEDGEMENTS

First, I would like to thank my mentor, Dr. Bill Rebeck, for training me to be an inquisitive researcher, supporting me and giving me these opportunities. Bill, you taught me many things, and I will always remember you telling me that I should be the most skeptical person in the room when presenting data. You are a remarkable mentor: patient, attentive, and exceptionally wise.

I would like to thank my parents, Andrej and Ruza, for believing in me and encouraging me to work hard. I thank you for your courage and strength, you made so many opportunities possible by changing our lives and moving to the United States.

I would like to thank my brother, Luka, for giving me everlasting support and love. Your dedication to your work always encouraged me and I still want to be just like you.

I would like to thank many friends for giving me confidence and support. Scott, thank you for the encouragement, your love keeps me grounded and focused. Diane, thank you for being my best friend, always making me laugh. Alexis and Missy, thank you both for your sound friendships, you are great true role models.

Also, I would like to thank my thesis committee: Dr. Timothy Mhyre, Dr. Italo Mocchetti, Dr. Dudley Strickland, and Dr. Zofia Zukowska. I sincerely thank you for your insight and attention. I would especially like to thank Dr. Dudley Strickland and members for his lab for collaborating with us on an important part of my thesis and giving me the opportunity to work in your lab over the past year.
This dissertation is dedicated to my parents,
Andrej and Ruža Počivavšek.

Zahvaljujem se vam za vso podporo,
Za vso moč,
Za vso modrost,
In za neizmirno ljubezen.

Rada vas imam
in sem predvsem zelo
hvaležna
ker ste mi vse
omogočili.
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Chapter I: Introduction

A. Alzheimer’s Disease and ApoE

Alzheimer’s disease (AD) is a debilitating neurodegenerative disorder characterized by memory loss. In the United States over 5 million individuals are living with AD and it is predicted to increase 3-fold as the population ages. Neuropathologically, AD is defined by the presence of extracellular amyloid plaques, intracellular neurofibrillary tangles and extensive neuronal loss in the central nervous system (CNS) (Selkoe 2001). Amyloid plaques are formed from accumulation of the amyloid beta (Aβ) peptide. This peptide originates from the cleavage of amyloid precursor protein (APP) by β-secretase and γ-secretase. Neurofibrillary tangles result from the accumulation of intracellular hyperphosphorylated tau deposits. Together these pathologies burden the brain and induce significant neuronal loss over time (Selkoe 2001).

The amyloid cascade hypothesis suggests that Aβ accumulation triggers progression of AD. Aβ builds up in the brain because of increased amyloidogenic processing of APP or decreased clearance of the toxic peptide. A chronic imbalance between Aβ production and clearance results in accumulation of amyloid plaques over time. A multistep cascade is then initiated that includes an astrocyte and microglial inflammatory response, oxidative stress, increased phosphorylation of tau and formation of tangles, and finally widespread neuronal lose (Hardy and Allsop 1991; Hardy and Selkoe 2002).
While most AD cases are sporadic, researchers have identified some genetic risk factors. One class of genetic risk factors is associated with early-onset familial AD, caused by an increased accumulation of Aβ. This class of genetic risk factors includes mutations to either APP itself or enzymes that form the secretase complexes that proteolytically cleave APP (presenilin-1 and presenilin-2) (Tanzi and Bertram 2005). Familial AD cases can have age of onset in the 40s, 50s or 60s, about 20 years before the age of onset for sporadic cases.

The second class of genetic risk factors for AD is involved in the late-onset form of the disease, which accounts for > 95% of cases. Apolipoprotein E (APOE) is the gene that has the strongest effect on the risk for AD (Weisgraber 1994). APOE is located on chromosome 19 and encodes a secreted protein of 299 amino acids. Single nucleotide polymorphisms in the APOE gene result in three common protein isoforms. These isoforms differ by the amino acids found at residues 112 and 158. ApoE3 has Cys-112 and Arg-158, ApoE4 has arginine at both positions, and ApoE2 has cysteine at both positions (Weisgraber 1994). APOE ε4 is associated with an increased risk of AD and APOE ε2 with decreased risk, compared to the most common form, APOE ε3 (Strittmatter et al. 1993). Various studies have shown that APOE ε4 is associated with an increased risk as well as earlier age of onset for developing AD (Corder et al. 1993). APOE e4 individuals have higher levels of Aβ in the brain (Rebeck et al. 1993), which may contribute to the poor prognosis of AD. On the other hand, the APOE ε2 allele has been associated with reduced relative risk and a delayed onset of AD (Corder et al. 1993).
ApoE has been found to associate with amyloid plaques and Aβ deposits as well as soluble Aβ in the CNS (Namba et al. 1991; Wisniewski and Frangione 1992; Wisniewski et al. 1993). One hypothesis suggests that the interaction between apoE and Aβ facilitates oligomerization of the peptide and formation of senile plaques (Bales et al. 2000; Bales et al. 1999). Another hypothesis suggests that apoE containing lipoproteins aid in clearing Aβ through apoE receptors (LaDu et al. 1994; Rebeck et al. 1993). Both of these hypotheses are supported by data in vitro; apoE4 promotes Aβ fibril formation to a greater extent than apoE2, whereas apoE2 promotes clearance of Aβ to a greater extent than apoE3 or apoE4 (Andersen and Willnow 2006).

Aside from AD, the APOE ε4 genotype may also be a risk factor for other CNS damages, such as stroke and traumatic brain injury (Alexander et al. 2007; Friedman et al. 1999; Isoniemi et al. 2006). These genetic associations have led numerous researcher to study the role of apoE in the CNS.

**B. ApoE: *What, Where and Why in the Brain?***

ApoE is a 34-kDa glycosylated protein that associates with lipoproteins and mediates trafficking of lipids throughout the circulatory system and between cells. In the periphery, apoE is synthesized by numerous cell types and is found associated with both very low-density lipoproteins and high-density lipoproteins. In the CNS, apoE is primarily produced by glia, including astrocytes (Grehan et al. 2001; Pitas et al. 1987) and activated microglia (Uchihara et al. 1995; Xu et al. 2000). ApoE is made and
secreted into the extracellular space, where it is associated with high-density lipoproteins through its amphipathic C-terminus. These high-density lipoproteins are important for redistribution of lipids after damage and clearance of lipids from the brain.

1. ApoE Receptors

ApoE is a ligand for low-density lipoprotein (LDL) receptor family members, binding them through its N-terminus. The LDL receptor family encompasses over ten receptors that endocytosis various ligands and function as cell surface signaling receptors (Herz and Bock 2002; May et al. 2005). In the CNS, several LDL receptor family members are expressed, including low-density lipoprotein receptor (LDLR), very-low density lipoprotein receptor (VLDLR), apolipoprotein E receptor 2 (ApoEr2), and LDL receptor-related protein-1 (LRP1). Neurons express ApoEr2, VLDLR, and LRP1 (Christie et al. 1996; Kim et al. 1996; Rebeck et al. 1993). Astrocytes and microglia express LDLR, LRP1, and VLDLR, but not ApoEr2 (Christie et al. 1996; Rebeck et al. 1993). The LDL receptor family in neurons is important for various processes, including neurite outgrowth (Narita et al. 1997; Postuma et al. 1998), neuronal calcium homeostasis (Bacskai et al. 2000; Levi et al. 2003; Ohkubo et al. 2001; Qiu et al. 2002), neuronal kinase activation (Arnaud et al. 2003; Bock and Herz 2003; Bock et al. 2003; Bock et al. 2004; Hoe et al. 2005; Qiu et al. 2004), and neuronal migration (Andrade et al. 2007; D'Arcangelo et al. 1999; Dulabon et al. 2000; Gotthardt et al. 2000; Trommsdorff et al. 1999). Little has been studied about the signaling properties of these receptors in glia.
2. **LDL Receptor Family Structural Characteristics**

The LDL receptor family is characterized by the presence of five distinct domains: i) ligand binding cysteine-rich repeats, ii) epidermal growth factor type cysteine-rich repeats, iii) YWTD domains that make a set of β-propellers, iv) one transmembrane domain, and v) at least one “NPxY” (Asn-Pro-any amino acid-Tyr) motif in the cytoplasmic tail (Herz and Bock 2002). Some receptors have an O-linked sugar domain that is present immediately extracellular to the transmembrane segment (Herz and Bock 2002).

Another common feature of the LDL receptor family members is that they all bind receptor-associated protein (RAP). Physiologically, RAP functions as a chaperone for these receptors, binding them in the endoplasmic reticulum and preventing premature interactions with ligands (Willnow et al. 1996). Pharmacologically, RAP has been used as a tool to prevent ligands from binding LDL receptor family members on the cell surface (Hoe et al. 2005; Koistinaho et al. 2004; Pocivavsek et al. 2009).

Clathrin-mediated endocytosis allows apoE receptors to internalize ligands. The rate at which receptors are endocytosed varies for the family members. For the CNS receptors, LRP is endocytosed fastest, with a half-life on the cell surface of 0.5 min. The surface half life of LDLR is about 4.8 min and those of ApoE2 and VLDLR are about 8 min (Li et al. 2001). These different times for endocytosis may reflect functional differences of these receptors: the receptors with shorter surface half-lives may have endocytic functions and those with longer half-lives may possess more
signaling functions. After endocytosis, ligands detach from the receptor and can be degraded; receptors can then be recycled to the cell surface. Trafficking of LDL receptor family members to and from the cell surface depends on interactions of adaptor proteins with the cytoplasmic tails of receptors. The short NPxY sequence on LDL receptor family members functions as a docking site for cytoplasmic adaptor proteins. Various adaptor proteins, including Disabled-1 (Dab1), FE65, JNK-interacting protein (JIP), and PSD-95, interact with the cytoplasmic tail of LDL receptor family members (Gotthardt et al. 2000; Trommsdorff et al. 1998).

3. **LDL Receptor Family Signaling Pathways**

Several recent studies have focused on the signaling properties of members of the LDL receptor family, specifically, LRP1, ApoEr2, and VLDLR (Herz 2001; May et al. 2005). VLDLR and ApoEr2 are both important for proper neuronal migration during development because they serve as receptors for the extracellular matrix molecule Reelin. This signaling requires the adaptor protein Dab-1 (Trommsdorff et al. 1999). LRP has been implicated in modulating NMDA receptor function (Bacskai et al. 2000; Hoe et al. 2005; Qiu et al. 2002). Proteolytic cleavage of LDL receptor family members has been shown to influence these intracellular interactions and affect cell signaling (Rebeck et al. 2006).

4. **ApoE Receptor Signaling and MAPK Cascades**

The cytoplasmic tails of ApoEr2 and LRP1 interact with c-Jun N-terminal kinase (JNK)-interacting protein (JIPs) (Stockinger et al. 2000). JIPs have been
identified as both inhibitors and activators of JNK signal transduction pathways (Dickens et al. 1997; Mooney and Whitmarsh 2004; Whitmarsh et al. 2001; Willoughby et al. 2003). JNK is part of the group of mitogen-activated protein kinases (MAPKs), along with ERK and p38. In neurons, our group found that apoE modulated the activation of two MAPK family members (Hoe et al. 2005; Hoe et al. 2006): extracellular signal-regulated kinase (ERK) was activated with apoE or apoE peptide treatment, while c-Jun N-terminal kinase (JNK) activation was reduced with apoE or apoE peptide treatment in primary neurons as well as in vivo (Hoe et al. 2005; Hoe et al. 2006). These effects were mediated through apoE receptors and require interactions with other cell surface molecules.

C. ApoE and Inflammation in the Brain

In the CNS, glial cells (microglia, astrocytes, and oligodendrocytes) support and nourish neurons, promoting their survival. For example, glial cells can release neurotransmitters and other signals regulating the strength of neuronal synapses or glial cells can sequester calcium, preventing its neurotoxic effects. In the healthy brain, microglia and astrocytes respond to stressful stimuli by secreting neuroprotective agents, helping to return the brain to homeostasis. However, with a more severe or chronic injury, these normal actions of glial cells can propagate neurodegeneration. Chronic activation of glia is detrimental to neurons as a variety of neurotoxic molecules such as nitric oxide (NO), reactive oxygen species (ROS), excitotoxins, and
pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-α) are over-produced (Boje and Arora 1992; Combs et al. 2001; Meda et al. 1995).

Microglia are resident macrophages in the brain and are important for mediating inflammation. Microglia, like macrophages, act as antigen presenting cells and promote inflammation by secreting various signaling molecules to secure homeostasis (Garden and Moller 2006). Morphologically, microglia change shape from a resting state to an activated state. Resting or quiescent microglia have long branching processes and a smaller cell body. The cell body of quiescent microglia does not move much, while its branches are continuously surveying the environment. Once microglia are activated, they undergo morphological changes including retraction of branches, expression of immunomodulatory proteins, secretion of cytotoxic agents, and secretion of pro-inflammatory signaling molecules. Activated microglia are large amoeboid shaped cells with phagocytic properties. These phagocytic cells move to the site of injury, engulf foreign particles, and secrete various pro-inflammatory agents that induce an inflammatory response in the brain and promote proliferation of microglial cells (Garden and Moller 2006).

Numerous studies have shown an increase in brain inflammation as another hallmark of AD brain pathology (Bales et al. 2000). Examination of postmortem brain tissue from patients with AD has revealed an increase in cytokines and chemokines (McGeer and McGeer 2001a; McGeer and McGeer 2001b). As Aβ accumulates extracellularly, activated astrocytes and microglia surround forming plaques (Koenigsknecht-Talboo et al. 2008; Uchihara et al. 1995). Activation of microglia and
astrocytes in response to Aβ has been observed \textit{in vitro} as well as \textit{in vivo} in animal models (Araujo and Cotman 1992; Bolmont et al. 2008; Meyer-Luehmann et al. 2008; Sondag et al. 2009). Glial cell activation leads to release of proinflammatory cytokines, chemokines, and other neurotoxic agents (Akiyama et al. 2000; Craft et al. 2005) that leads to neuronal cell dysfunction.

1. \textit{ApoE Regulation of Inflammation in the Brain}

Increasing evidence suggests that apoE modulates the CNS inflammatory response. Initial studies showed that apoE knockout animals had increased brain inflammation after injury, compared to wild-type controls (Lynch et al. 2001). ApoE isoform specific differences in the response of glial cells have been demonstrated in animal models expressing different apoE isoforms (Vitek et al. 2007). Across paradigms, the apoE2 isoform demonstrates greater anti-inflammatory properties than the apoE3, which has greater anti-inflammatory properties than apoE4. Application of exogenous apoE or apoE mimetic peptides formed from the receptor-binding region of apoE, modulate the inflammatory response (Laskowitz et al. 1997; Laskowitz 2000; Laskowitz et al. 1998; Laskowitz et al. 2001; Lynch et al. 2001; Lynch et al. 2003; Mace et al. 2007). However, little work has been done to investigate the mechanisms involved in apoE-dependent immunomodulation.

2. \textit{JNK and Glial Activation}

The MAPK family, including p38, ERK, and JNK, has been implicated in mediating glial activation (Bhat et al. 1998; Han et al. 2002; Pyo et al. 1998; Xie et al.
The JNK family includes three isoforms, termed JNK1, JNK2, and JNK3. Upstream MAPK kinases (MAP2K) and MAPK kinase kinases (MAP3K) activate JNKs. Scaffolding proteins are responsible for bringing together the upstream and downstream kinases in close proximity and ensuring proper activation and signal transduction (Waetzig et al. 2005). JIPs are examples of these scaffolding proteins, which are important for activating all three JNKs and are of interest here because there are JIP binding sites on LDL receptor family members.

Upon activation, JNK can bind and phosphorylate various substrates in different parts of the cell including the cytoskeleton, mitochondria, and the nucleus. JNKs activate transcription factors in the nucleus such as c-Jun. In the CNS, JNKs are important for activating pro-apoptotic target genes (Putcha et al. 2003). JNK2 and JNK3 move to the nucleus rather rapidly and are thus thought to exert activation of transcription factors. JNK1 remains constitutively present in the nucleus and therefore not effective at targeting activation of transcription factors. JNK-induced activation of c-Jun leads to degenerative actions of JNKs. Knocking out c-jun is lethal developmentally (Hilberg et al. 1993), although inhibiting phosphorylation of c-jun by altering serine residues 63 and 73 with alanines preserves a normal phenotype (Behrens et al. 1999). Pharmacological inhibition of c-jun can been accomplished by preventing activation of JNK.

Evidence in vitro and in vivo suggests that JNK activation contributes to neurodegeneration in AD (Okazawa and Estus 2002). For example, JNK inhibition overcomes Aβ induced neurotoxicity in cultured neurons (Bozyczko-Coyne et al. 2001;
Troy et al. 2001). However, little attention has been given to JNK activation in glial cells during neurodegenerative processes.

Microglial cells express all three JNK isoforms. Various inflammatory stimuli, including the endotoxin lipopolysaccharide (LPS) and TNF-α, induce JNK activation in primary rodent microglia (Hidding et al. 2002). JNKs are involved in the enlargement of the microglial cell body in addition to increased expression of pro-inflammatory genes such as cyclooxygenase (COX)-2, TNF-α, interleukin (IL)-6, and monocyte chemotactic protein (MCP)-1 (Waetzig et al. 2005). Taken together, inhibition of JNK activation may overcome microglial inflammation (Waetzig and Herdegen 2004). Compounds that inhibit JNK have entered clinical trials to treat peripheral diseases like leukemia and other cancers (Bogoyevitch and Arthur 2008; Manning and Davis 2003) and could be therapeutic targets to overcome the inflammatory response in CNS diseases such as AD.

3. **JNK and AP-1 Transcription Factor**

JNK phosphorylation leads to the activation of c-Jun and activating protein 1 (AP-1), a transcription factor composed of heterodimers of several family members, which includes Jun and Fos. As with most transcription factors, AP-1 proteins function in several ways including 1) inducing transcription; 2) suppressing transcription; 3) modulating transcription by interacting with other transcription factors; and 4) non-transcriptional effects outside of the nucleus (Herdegen and Waetzig 2001). As inducers of transcription, AP-1 proteins bind to AP-1 consensus motifs (5’-TGAG/CTCA-3’) and orchestrate the start of transcription. On the other
hand, AP-1 proteins can also bind the regulatory sequence without activating transcriptional machinery. In this case, the transcription factor may be suppressing transcription. AP-1 proteins can also modulate transcription by associating with other transcription factors, for example steroid-hormone-complexes. This interaction prevents both transcription factor complexes from binding DNA and inducing transcription (Herdegen and Waetzig 2001).

D. Regulation of ApoE Expression

ApoE expression is regulated by neuronal injury and glial activation. ApoE is increased in the CNS after neuronal injury (Messmer-Joudrier et al. 1996; Page et al. 1998; Petegnief et al. 2001; Poirier 1991; Stoll 1989) and decreased when macrophages or glia are activated by an endotoxin such as LPS (Dory 1993; Gafencu et al. 2007; Menju et al. 1989; Mouchel et al. 1995; Saura et al. 2003; Zuckerman and O'Neal 1994).

Although apoE is primarily produced by glia (Grehan et al. 2001; Pitas et al. 1987; Uchihara et al. 1995; Xu et al. 2000), expression of apoE in neurons has been reported after neuronal injury (Huang et al. 2004). Transcription of APOE may be differentially regulated by cells in response to their cholesterol transport needs. The APOE gene has a molecular sensor, the liver X receptor (LXR), which is a nuclear receptor that regulates cholesterol homeostasis and mediates transcription. Agonists of LXR increase APOE transcription (Eckert et al. 2007). The differences in APOE
mRNA expression among cell types strongly suggest that apoE has various and specific functions to each cell type.

1. **APOE Gene Characteristics**

The human APOE gene is located on chromosome 19 (Olaisen et al. 1982). At the 5’-end of chromosome 19 there is an apoE/apoCI/apoCIV/apoCII gene cluster. The mouse APOE gene is located on chromosome 7 and both human and mouse genes have four exons and three introns (Maloney et al. 2007). The coding sequence of APOE is well conserved among various species, with 70% homology between mouse and human APOE (Rajavashisth et al. 1985). The mouse and human sequences are also > 90% homologous in the promoter region closest to the transcription start site (150 base pairs of +1). Upstream of this region, homology is reduced to < 40% (Maloney et al. 2007). Thus, most effort has focused on examining gene regulation in this region closest to the transcription start site.

The TATAA box has remained preserved between mouse and human apoE gene sequences. Importantly, several transcription factor binding sites are conserved among species in the proximal -120/+20 promoter region including AP-1, GC box, AP-2, glucocorticoid receptor (GR), nuclear factor (NF)-kB, and stimulatory protein (SP)1 (Maloney et al. 2007).

2. **ApoE Protein Characteristics**

The amino-terminal domain of apoE is a four-helix bundle, composing the receptor-binding domain. The carboxyl-terminal is highly alpha-helical and contains
the lipid-binding domain (Weisgraber and Mahley 1996). The LDL receptor family
binding domain is located in helix 4, residues 136-150, and is enriched in lysine and
arginine residues (Weisgraber 1994). The human isoforms apoE3 and apoE4 bind the
LDL receptor family members with similar high affinity, while apoE2 binds with
approximately 50 times weaker affinity (Weisgraber et al. 1982). Because of its
arginine at position 112, apoE4 preferentially binds large lower-density lipoproteins,
while apoE3 and apoE2 preferentially bind smaller, cholesterol-rich high-density
lipoprotein particles (Hatters et al. 2006). X-ray structures shows that Arg112 on
apoE4 affects conformation of the Arg61 residue and a domain interaction between the
carboxyl-terminal and amino-terminal domains of apoE. In apoE4, Arg61 interacts
strongly with an acidic Glu255 on the carboxyl-terminal, an interaction that does not
occur in apoE3 or apoE2 (Dong and Weisgraber 1996). In all species, other than
humans, a threonine is present at the position equivalent to Arg61, eliminating the
interaction between the amino and carboxyl terminal domains. Thus, in other species,
like mice, apoE does not show domain interactions and instead behaves functionally
like apoE3 (Hatters et al. 2006).

E. Impact of ApoE Isoforms on Brain ApoE Levels

The underlying molecular mechanisms attributing apoE4 as a risk factor for
various CNS diseases remain unclear. Because of the arginines at amino acid positions
112 and 158, apoE4 has major structural characteristics that distinguish it from apoE3
or apoE2. These structural changes diminish the stability of apoE4 (Hatters et al.
and cause apoE4 to bind low-density lipoprotein particles more avidly, thus clearing apoE4 more readily (Raffai et al. 2001). Furthermore, in the CNS recent evidence suggests that glial cells preferentially degrade apoE4 (Riddell et al. 2008). To study differences in apoE isoforms in vivo, apoE targeted replacement mice were designed. In these mice, the mouse APOE gene has been replaced with the human APOE ε2, ε3, or ε4 alleles. These animal models are invaluable for studying the impact of genotype on either normal or diseased CNS function in vivo. Several recent reports show that levels of apoE protein are differentially expressed among these targeted replacement mice. Decreased levels of brain apoE have been reported in apoE4 targeted replacement mice (Riddell et al. 2008; Vitek et al. 2007). These findings suggest that the apoE isoform associated risk may be related to the amount of apoE protein.

F. Overall Significance and Aims

This thesis will address five main questions: 1) How can we induce glial activation & measure the inflammatory response; 2) Do LDL receptor ligands inhibit these effects; 3) What are the signaling pathways that mediate these effects; 4) Which LDL receptor family members are responsible for these effects; and 5) How can we modulate the glial inflammatory response as well as regulate levels of apoE?

APOE is the strongest genetic risk factor for late onset AD. One possible function of apoE in the brain is that it modulates the inflammatory processes associated with AD pathological changes, perhaps through the LDL receptor family (LaDu et al.
2000; LaDu et al. 2001). We aim to elucidate the signaling cascades activated in glial cells by this family of receptors. Our goal is to modulate the glial inflammatory response, as well as regulate levels of apoE, as one approach to limiting CNS inflammation.
CHAPTER II: Low Density Lipoprotein Receptors Regulate Microglial Inflammation Through C-Jun N-Terminal Kinase

A. Brief Overview

Inflammation is a hallmark of many CNS diseases, including AD. Increasing evidence suggests that apoE downregulates CNS inflammation: apoE modulates the inflammatory response of microglia in an isoform specific manner and application of apoE or apoE mimetic peptides reduces the inflammatory response (Aderem and Ulevitch 2000; Laskowitz et al. 1997; Laskowitz et al. 2000; Laskowitz et al. 1998; Laskowitz et al. 2001; Lynch et al. 2001; Lynch et al. 2003; Mace et al. 2007; Ophir et al. 2005; Vitek et al. 2007).

While apoE modulates the CNS inflammatory response, apoE expression itself is also regulated by neuronal injury and glial activation. ApoE is upregulated in the CNS after neuronal injury (Poirier et al. 1991) and down-regulated when macrophages or glia are activated by LPS (Dory 1993; Gafencu et al. 2007; Menju et al. 1989; Mouchel et al. 1995; Saura et al. 2003; Zuckerman and O'Neal 1994). ApoE is expressed mainly in glial cells (Boyles et al. 1985; Murakami et al. 1988) and maintenance of apoE is vital to maintain cholesterol transport required for membrane repair and recycling in the CNS.

Though the mechanisms by which apoE modulates the CNS immune responses have not been elucidated, some evidence suggests that members of the LDL receptor family can modulate the glial inflammatory response (LaDu et al. 2000; LaDu et al. ...)
2001; Laskowitz et al. 2001). The aims of our present study were to address how LDL receptors regulate the CNS inflammatory response in BV2 microglia and primary mouse microglia. For these studies, we used an apoE peptide (EP) that consisted of a tandem repeat of the receptor-binding domain; this peptide binds apoE receptors and activates signaling processes in neurons (Hoe et al. 2005; Hoe et al. 2006). We studied mitogen-activated protein kinase (MAPK) family members, a group implicated in microglial activation (Bhat et al. 1998; Han et al. 2002; Pyo et al. 1998; Xie et al. 2004) and neuronal apoE signaling (Hoe et al. 2005; Hoe et al. 2006). Microglial activation was monitored by LPS-induced accumulation of nitric oxide (NO) and reduced apoE expression. Inhibition of the MAPK family member JNK by EP proved to be essential to overcome LPS-induced microglial inflammatory responses.

B. MATERIALS AND METHODS

**BV2 Microglial Cell Culture:** BV2 microglia were provided by Dr. Richard Banati (Max-Planck-Institute of Psychiatry, Martinsried/Munich, Germany) (Blasi et al. 1990). Cells were maintained in Opti-MEM (Invitrogen, Carlsbad, CA) with 5% fetal bovine serum (FBS, Invitrogen) in a 5% CO₂ incubator. For experiments, cells were plated in 24-well plates at 7.5 X 10⁴ cells / well. Plated cells were grown in Opti-MEM with 5% FBS overnight and then the medium was changed to serum-free Opti-MEM for 1 hr. After 1 hr, the medium was replaced with serum-free Opti-MEM containing either control PBS or experimental agents.
Primary Microglial Cell Culture:  Primary mouse microglial cell cultures were prepared from postnatal day 1 Swiss-Webster mouse pups using a slightly modified method from that of Su et al. (2007). Mixed glial cultures were grown to confluency and microglia were harvested by shaking the flasks at 100 rpm for 1 hr at 37°C. Microglial were resuspended in minimum essential media (MEM, Invitrogen) supplemented with 10 % FBS, 1% L-glutamine (Invitrogen), 1% sodium pyruvate (Invitrogen), 1% Pen/Strep (Invitrogen), and 1% Fungizone (Invitrogen) and plated at a density of 1 x 10^5 cells/ml on glass coverslips. Microglial cell purity was confirmed to be >95% using immunofluorescence for OX42 (microglial marker, Serotec, Raleigh, NC) and DAPI (Vector Laboratories, Burlingame, CA) nuclear counterstain (Supplemental Fig. 1A). For nitrite assay experiments, cells were plated in 96-well plates at 2.5 X 10^4 cells / well. For Western blot analysis experiments, cells were plated in 24-well plates at 12.5 X 10^4 cells / well. Plated cells were grown in supplemented media overnight and then the medium was replaced with serum-free Opti-MEM containing either control PBS or experimental agents.

Antibodies:  Phosphorylation site-specific antibodies were used against phospho-p38 (Thr180/Tyr182), phospho- ERK (Thr202/Tyr204), and phospho-JNK (Thr183/Tyr185) (Cell Signaling Technologies, Beverly, MA). Phospho-ERK antibody detected levels of both p42 (Erk2) and p44 (Erk1) MAP Kinases when phosphorylated. Phospho-JNK antibody detected levels of p46 (JNK1) and p54 (JNK2) kinases when phosphorylated. Total ERK and JNK antibodies (Cell Signaling Technologies) detected total levels of ERK and JNK proteins, including both ERK
proteins (p42 and p44) and both JNK proteins (p46 and p54). ApoE was detected by rabbit polyclonal antibody against rodent apoE (Abcam, Cambridge, MA). Rabbit polyclonal antibody was used to detect iNOS (BD Biosciences Pharmigen, San Diego, CA). From the same blots, β-actin (Abcam) was detected by monoclonal antibody to ensure equal protein levels in each lane. To detect LDL receptors, we used rabbit polyclonal antibodies against LDLR (Irene) and LRP (RRR) provided by Dr. Guojun Bu (Washington University, St. Louis, MO). VLDLR was detected by mouse monoclonal antibody (5F3), provided by Dr. Dudley Strickland (University of Maryland, Baltimore, MD). OX42 (Serotec, Raleigh, NC) was used to stain primary microglia.

**Chemicals:** The apoE-derived peptide<sub>(141-149)</sub> (EP), consisting of a duplicated sequence of apoE amino acids 141 through 149, was synthesized by Johns Hopkins University of Medicine (Biosynthesis and Sequencing Facility, Baltimore, MD) (Hoe et al. 2005). Recombinant RAP was a generous gift from Dr. Dudley Strickland (University of Maryland) and Dr. Guojun Bu (Washington University). Wortmannin (Sigma) inhibited PI3K/AKT, PD98059 (Sigma) inhibited ERK, and SP600125 (Invitrogen) inhibited JNK. Phosphatase inhibitor cocktails (Sigma) and protease inhibitor (Sigma) were used in cell lysis buffer. LPS was purchased from Calbiochem (San Diego, CA). Human recombinant α2M (Athens Research, Athens, GA) was activated in 100mM methylamine (Sigma) at 1mg/ml as stock solution. FITC-α2M* was made with EZ-Label™ FITC Protein Labeling Kit (Pierce Biotechnology, Rockford, IL).
VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) was used to fluorescently label nuclei.

**Western Blot Analyses:** For analysis of secreted molecules, conditioned media was collected from cultured cells. Cell associated proteins were harvested in ice-cold lysis buffer containing phosphatase inhibitor cocktails and protease inhibitor. Proteins were separated under denatured and reduced conditions using Tris-glycine SDS-polyacrylamide gel electrophoresis (Biorad, Hercules, CA). Separated proteins were detected on poly(vinylidifluoride) membranes incubated with primary antibodies. Immunoreactivity was detected using horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) visualized by enhanced chemiluminescence detection film. Band density was determined using Quantity One 1-D analysis software (Biorad).

**Nitrite Quantification:** The production of NO was assessed as the accumulation of nitrite from the spontaneous oxidation of NO in serum-free cell conditioned media after 24 hrs and 48 hrs. Accumulation of nitrite was quantified using a colorimetric reaction with Griess reagent (Invitrogen). Absorbance was measured at 570nm by spectrophotometry.

**Statistical Analysis:** All experiments were repeated a minimum of three times. The data were analyzed using ANOVA with Graphpad Prism 4 software, using Newman-Keuls Multiple Comparison Test for posthoc analysis with significance determined at a P value of <0.05.
C. RESULTS

Effects of microglial cell stimulation on nitric oxide production and apoE production

The Toll-like receptor 4 (TLR4) is traditionally considered the primary receptor for LPS (Lien et al. 2000). Upon receptor activation, LPS induces intracellular signaling cascades, downstream synthesis of inducible nitric oxide synthase (iNOS) and release of NO in microglia (Corradin et al. 1993). For our experiments, mouse microglial BV2 cells and primary mouse microglia were treated with 100 ng/ml LPS in serum-free media. LPS treatment caused a significant increase of NO from BV2 cells at 24 hrs and 48 hrs (Fig. 1A). BV2 cells treated with LPS accumulated 42 ± 2 µM nitrite at 24 hrs and 52 ± 4 µM nitrite at 48 hrs, compared to 4 ± 1 µM and 7 ± 2 µM nitrite from untreated cells. Expression of iNOS was readily detected by Western blot analysis of BV2 cells treated with LPS for 24 and 48 hrs, but undetected in untreated cells (Fig. 1B). Similarly, in primary microglia, LPS caused a significant increase in NO at 24 hrs (Fig. 1E). Microglia treated with LPS accumulated 6 ± 1 µM nitrite compared to 0.3 ± 0.1 µM nitrite from untreated cells. Expression of iNOS was detected by Western blot analysis in microglia treated with LPS for 24 hrs, but undetected in control cultures Fig. 1F).

LPS also reduced endogenous apoE in cell lysates and conditioned media of BV2 cells (Fig. 1C) and primary microglia (Fig. 1G). Total protein levels of β-actin remained unchanged. Quantification of Western blot analysis showed that apoE levels
in the BV2 cell lysate and conditioned media significantly decreased with LPS stimulation at 12 hrs, 24 hrs, and 48 hrs when compared to apoE expression in unstimulated cells at the same times (Fig. 1D). LPS significantly reduced cellular apoE levels by 39% at 12 hrs, 52% at 24 hrs, and 69% at 48 hrs, compared to control cells. ApoE levels in the conditioned media were reduced similarly (22% at 12 hrs, 37% at 24 hrs, and 52% at 48 hrs) (Fig. 1D). Western blot analysis of primary microglia was quantified showing that apoE levels in the cell lysate and conditioned media were also significantly decreased with LPS stimulation at 24 hrs when compared to apoE expression in unstimulated cells (Fig. 1H). Cellular apoE levels in primary microglia were decreased 27% with LPS treatment for 24 hrs. ApoE levels in the conditioned media were decreased 42% with LPS treatment (Fig. 1H). Thus, activation of both BV2 cells and primary microglia by LPS increased iNOS levels and production of NO, and decreased levels of apoE.

**ApoE Peptide Attenuates LPS Induced Inflammatory Responses**

Various apoE peptides, formed from the receptor-binding region of apoE, have anti-inflammatory properties (Laskowitz et al. 2001). In this study, we used the apoE mimetic peptide, EP, consisting of a tandem repeat of the nine amino acid receptor-binding domain of apoE. We hypothesized that EP could attenuate the LPS induced nitrite accumulation and reduction of apoE expression. Preliminary studies showed that pretreatment of cells with EP was not necessary for its effects (data not shown); therefore EP treatment was performed at the same time as LPS stimulation. BV2
microglia were treated with various doses of EP, ranging from 100 nM to 5 µM. EP modulated LPS-induced nitrite production optimally at doses of 1 µM or higher. BV2 cells stimulated with LPS accumulated 33 ± 2 µM nitrite at 24 hrs. Cultures treated with 1 µM EP and LPS accumulated 24 ± 2 µM nitrite, a significant 27% decrease compared to cells treated with LPS alone. Cultures treated with 5 µM EP and LPS accumulated 17 ± 1 µM nitrite, a significant 48% decrease compared to cells treated with LPS alone (Fig. 2A). Thus, EP counteracts the effects of LPS on nitrite production.

To test whether BV2 cells have functional receptors of the LDL receptor family, FITC-labeled activated alpha-2-macroglobulin (α2M*), a ligand for several members of this family, was applied and detected intracellularly by fluorescence microscopy (Supplemental Fig. 2A). Receptor associated protein (RAP) was used as a tool to block ligand binding to the receptors. Cells treated with RAP were unable to endocytose FITC-labeled α2M*, proving that RAP was active (data not shown). We used RAP to block EP from binding the receptors and to test whether EP was reducing nitrite accumulation through interactions with the LDL receptor family. BV2 cells treated with 50 nM RAP, 5 µM EP, and LPS accumulated significantly higher levels of nitrite compared to cells treated with 5 µM EP and LPS alone (39 ± 2 µM compared to 22 ± 2 µM) (Fig. 2B). Cells were treated with RAP and LPS alone as a control and those cells accumulated approximately the same amount of nitrite as LPS stimulated
cells (34 ± 2 µM nitrite). Thus, LDL receptor family mediated the inhibitory effect of EP on nitrite production.

Similar results were observed in primary microglia. LPS increased nitrite levels, and this effect was partially blocked by EP (8 ± 0.3 µM compared to 12 ± 1 µM) (Fig. 2E). Microglia treated with 50 nM RAP, 5 µM EP, and LPS accumulated significantly higher amounts of nitrite compared to cells treated with 5 µM EP and LPS alone (12 ± 1 µM compared to 8 ± 0.3 µM). As a control, microglia were treated with RAP and LPS alone and those cultures accumulated similar amounts of nitrite as LPS stimulated cells (11 ± 2 µM). The data from both primary microglia and BV2 cells support the idea that LDL receptors mediate inhibitory effects of EP on nitrite production.

We also tested whether EP altered the effect of LPS on apoE levels. These experiments were done in both BV2 cells and primary microglia. For these experiments, apoE expression in the cell lysate was analyzed by Western blot (Fig. 2C for BV2 cells; 2F for primary microglia) and quantified (Fig. 2D for BV2 cells; quantification not shown for primary microglia). Cells treated with 1 µM EP alone did not have a significant change in endogenous apoE expression compared to controls. Cells stimulated with LPS had significantly less apoE than control (50 ± 4% of control in BV2 cells; 66 ± 5% of control in primary microglia), consistent with Fig. 1. Cells treated with 1 µM EP and LPS had significantly more apoE than cells treated with LPS.
alone (80 ± 5% of control in BV2 cells; 85 ± 6% in primary microglia). Thus, EP treatment attenuated the reduction of endogenous apoE caused by LPS.

To examine whether members of the LDL receptor family mediated the effect of EP, we again used the receptor blocker RAP. When BV2 cells were treated with RAP alone, there was no significant change in apoE levels (88 ± 4% of control); LPS also still reduced apoE levels in the presence of RAP (58 ± 3% of control). However, in the presence of RAP, EP no longer reversed the effect of LPS (40 ± 2% of control). These data indicate that EP attenuates LPS induced reduction in apoE expression via the LDL receptor family.

**ApoE Peptide Inhibits JNK Activation**

We have shown that EP attenuates nitrite production and changes in endogenous apoE, caused by LPS in BV2 cells and primary microglia. To examine the signaling pathways that may mediate these responses, we continued our research in BV2 microglia and studied the activation of kinases JNK and ERK, both implicated in LPS-induced signaling cascades (Bhat et al. 1998; Han et al. 2002; Pyo et al. 1998; Schumann et al. 1998). Activation of JNK and ERK was initially studied from 5 min to 60 min and cells were treated with either 100 ng/ml LPS or 1 µM EP. LPS caused an increase in phospho-ERK levels, while levels of total ERK remained unchanged (Fig. 3A). LPS also caused a dramatic increase in phospho-JNK levels and levels of total JNK remained unchanged (Fig. 3A). Quantification of the Western blot analysis showed that LPS induced significant time-dependent increases in both phospho-ERK
and phospho-JNK levels (Fig. 3C). Levels of phospho-ERK and phospho-JNK activation were also analyzed for cultures stimulated with EP from 5 min to 60 min. EP caused an increase in phospho-ERK levels, while total levels of ERK remained unchanged (Fig. 3B). In contrast, EP induced a decrease in phospho-JNK, while levels of total JNK remained unchanged (Fig. 3B). Together, these data show that EP caused a significant time-dependent increase in phospho-ERK and a time-dependent decrease in phospho-JNK (Fig. 3C). Thus, LPS and EP have qualitatively similar effects on ERK activation, but strikingly different effects on JNK activation (Fig. 3C).

We asked how these signaling pathways were affected with simultaneous EP and LPS treatment. ERK and JNK phosphorylation was examined 1 hr after treatments because the most pronounced signaling effects were seen at 1 hr (Fig. 3). As before, treatment of BV2 cells with LPS for 1 hr increased phospho-ERK and phospho-JNK expression, while levels of total ERK and JNK remained unchanged (Fig. 4A). LPS-treated cells had 425 ± 24% of control levels in phospho-ERK and 990 ± 130% of control levels of phospho-JNK. Simultaneous treatment of BV2 cells with EP and LPS for 1 hr induced an even greater increase in phospho-ERK (596 ± 5% of control) and a significant decrease in phospho-JNK (532 ± 53% of control) levels when compared to cultures treated with LPS alone (Fig. 4B). To test whether apoE receptors were involved in the effects of EP on signaling, we again used the inhibitor RAP. Treatment of cultures with RAP, EP and LPS showed an increase in phospho-ERK levels (393 ± 44% of control) and an increase in phospho-JNK levels (1012 ± 89% of control) when compared to control cultures. RAP treatment prevented EP from potentiating LPS
induced phospho-ERK activation and prevented EP from attenuating LPS induced phospho-JNK activation (Fig. 4B). Thus, EP acts via the LDL receptor family to induce a potentiation of LPS-induced ERK activation and an attenuation of LPS-induced JNK activation.

**Inhibition of JNK Prevents LPS Induced Inflammatory Response**

The data in Figs. 3 and 4 show that activation of JNK and ERK can be modulated by EP in BV2 microglia. We next asked whether various kinases were involved in LPS-induced nitrite accumulation and apoE reduction using various inhibitors. We used inhibitors to the MAPK pathway family members, JNK and ERK, and an inhibitor to the phosphoinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway, implicated in regulating cellular inflammatory responses (Cantley 2002). Cells were treated with doses of JNK inhibitor (SP600125), ERK inhibitor (PD98059), and PI3K/Akt inhibitor (Wortmannin). The lowest dose used for each inhibitor blocked phosphorylation of its respective kinase at 1 hr (data not shown). Previous reports implicated JNK activation in iNOS expression and NO accumulation (Moon et al. 2007; Pawate and Bhat 2006) and our findings confirmed these results. Cells treated with LPS and SP600125 accumulated significantly less nitrite in a dose dependent manner when compared to cultures treated with LPS alone (Fig. 5A). Cells treated with PD98050 or Wortmannin showed no attenuation of the LPS induced nitrite response (Fig. 5A). Further, SP600125 blocked the increase in iNOS accumulation caused by LPS (Fig. 5B). In cells treated with low doses of SP600125 (0.1 µM and 1
µM) and LPS, iNOS levels remained equal to control cells treated with LPS alone. Cells treated with 10 µM SP600125 and LPS treatment showed a 20 % reduction in iNOS expression compared to cells treated with LPS alone. At higher doses of SP600125 (25 µM and 50 µM), iNOS expression was reduced by nearly 100 % compared to cells treated with LPS alone. The reduction in iNOS levels and nitrite levels occurred with the same doses of SP600125 (between 1 µM and 10 µM SP600125). These data show that JNK activation, but not ERK or PI3K/AKT activation, is important for LPS induced NO accumulation in BV2 cells.

We next asked whether the effects of EP were also mediated by the JNK pathway. We used suboptimal doses of both SP600125 (1µM and 5µM) and EP (1µM or 5µM) to test whether they would act synergistically on JNK activation and LPS-induced nitrite accumulation. As before, BV2 cells stimulated with LPS and treated with 1µM EP or 5µM EP had a significantly lower amount of nitrite accumulation than cells stimulated with LPS alone (Fig. 5C). Cells stimulated with 1µM SP600125 accumulated 30% (30 ± 2 µM) less nitrite than cells treated with LPS alone; cells stimulated with 1µM EP alone accumulated 21% (34 ± 2 µM nitrite) less nitrite than cells treated with LPS alone. Cells treated with 1µM SP600125 and 1µM EP alongside LPS stimulation accumulated 58% (18 ± 1 µM) less nitrite than cells treated with LPS alone and significantly less than cells treated with either drug alone. Further, cells treated with a higher dose of EP (5 µM) and 1 µM SP600125 also accumulated significantly less nitrite compared to cells treated with LPS alone (65% less; 15 ± 1
µM). Thus, EP and SP600125 can additively suppress LPS-induced nitrite accumulation. Western blot analysis indicated that iNOS levels were similarly decreased with a combinational treatment of EP and SP600125 (Fig. 5D).

We tested whether these mechanisms defined in BV2 cells also occurred in primary microglia. Similar to Fig. 2E, primary microglia stimulated with LPS and treated with 1 µM EP had a significant 25 % reduction in nitrite accumulation (6 ± 0.1 µM) than cells stimulated with LPS alone (8 ± 0.3 µM) (data not shown). Primary microglia stimulated with 1 µM SP600125 accumulated 75 % (2 ± 0.1 µM nitrite) less nitrite than cells treated with LPS alone. Cells treated with 1 µM SP600125 and 1 µM EP alongside LPS stimulation accumulated 89 % (1 ± 0.03 µM nitrite) less nitrite than cells treated with LPS alone, which was significantly less nitrite compared to cells treated with either drug alone. These effects of EP on nitrite levels in primary microglia were paralleled by effects on decreasing iNOS levels (data not shown).

Finally, to determine whether JNK was also involved in the down-regulation of apoE by LPS, we tested the effects of SP600125 on apoE levels after stimulation with LPS. For these experiments, apoE levels in BV2 cell lysates were analyzed by Western blot (Fig. 5E) and quantified (Fig. 5F). As before, LPS decreased apoE levels to 46 ± 5% of control (Fig. 5E & 5F). Cells treated with LPS and SP600125 showed a significant attenuation of the decrease in apoE (93 ± 7% of control). SP600125 alone increased apoE levels, suggesting that there is also regulation of apoE by JNK under normal conditions in cell cultures. Cells treated with the other kinase inhibitors,
PD98059 and Wortmannin, did not show an attenuation of LPS induced decrease in apoE expression. Thus, as with NO production, the effect of LPS on reducing apoE levels depended on the JNK signaling pathway.

D. DISCUSSION

Our current study demonstrates that apoE-induced anti-inflammatory properties involve MAPK signaling pathways that can be promoted through the activation of LDL receptors. This work is consistent with reports that apoE has anti-inflammatory properties in the CNS (Laskowitz et al. 1997; Laskowitz et al. 2000; Laskowitz et al. 1998; Laskowitz et al. 2001; Lynch et al. 2001; Lynch et al. 2003; Mace et al. 2007). Our data show that an apoE mimetic peptide, EP, attenuated LPS-induced inflammatory responses by modulating JNK activation. In microglia LPS activation of JNK increased NO and decreased apoE, the two inflammatory responses that we studied. We showed that EP treatment decreased JNK through the LDL receptor family and counteracted LPS-induced inflammatory responses through this modulation. Taken together, we developed a model of apoE receptor immunomodulatory signaling (Fig. 6), suggesting that the LDL receptor family regulates the microglial inflammatory response by suppressing JNK activation.

Several studies have implicated MAPK activation in microglia treated with various stimuli. LPS induces activation of all three major classes of MAPKs: p38, JNK, and ERK (Bhat et al. 1998; Han et al. 2002; Pyo et al. 1998; Xie et al. 2004). Our study confirmed these previous reports showing LPS-induced activation of p38
(data not shown), ERK and JNK (Fig. 3) in BV2 microglia. Our previous work in neurons showed EP-induced an increase in ERK activation and a decrease in JNK activation (Hoe et al. 2005). In microglia, we found that EP affects ERK and JNK activation, but does not alter p38 activation (data not shown). One potential mechanism through which MAPKs might contribute to microglial activation is through effects on NO generation (Chao et al. 1992; Saha and Pahan 2006; Simmons and Murphy 1992). Previous work showed that JNK is the major regulator of iNOS expression in primary astrocytes (Pawate and Bhat 2006). Our research focused primarily on the effects on JNK activation because our data show that specific JNK inhibitor (SP600125) inhibited iNOS and nitrite accumulation in a dose-dependent manner, implicating JNK as an important mediator of LPS-induced NO production, a finding that is in line with a recent study in BV2 cells (Moon et al. 2007). These findings support the idea that apoE protects against inflammatory signaling and NO production via inhibition of the JNK pathways.

LPS also decreased endogenous apoE expression in BV2 microglia and primary microglia. There are reports of LPS-induced down regulation of apoE gene and protein expression in macrophages (Dory 1993; Gafencu et al. 2007; Menju et al. 1989; Mouchel et al. 1995; Saura et al. 2003; Zuckerman and O'Neal 1994) or in mixed glial cultures and microglia (Saura et al. 2003). However, the mechanism by which LPS down regulates apoE expression in microglia has not been elucidated. Our work suggests that apoE regulation depends on JNK signaling. Treatment of microglia with EP prevented the LPS-induced decrease of apoE. Further, we found that inhibition of
JNK by specific inhibitor (SP600125) prevented LPS-induced reduction of endogenous apoE. Treatment of BV2 cells with ERK inhibitor (PD98059) or PI3K/Akt inhibitor (Wortmannin) did not inhibit NO production. These findings support the idea that apoE protects against inflammatory signaling and NO production via inhibition of the JNK pathways.

LPS-induced JNK activation leads to c-Jun phosphorylation and activation of AP-1 transcription factors (Hidding et al. 2002). A recent study suggests LPS treatment induces both AP-1 and NFκB to bind the apoE promoter and interfere with promoter activity resulting in repression of apoE gene expression (Gafencu et al. 2007). However, in another study, LPS-induced down regulation of apoE was not attenuated with inhibitors of NFκB (Saura et al. 2003), suggesting that factors besides NFκB regulate apoE expression. Our findings that JNK inhibition is important to overcome LPS-induced down regulation of apoE support a role for JNK in regulating apoE expression, perhaps by regulating AP-1 activation.

We showed that EP significantly inhibited JNK in a time-dependent manner and attenuated the LPS-induced inflammatory response in microglia. The effects of EP were found to be mediated by members of the LDL receptor family. This family has previously been implicated in modulating the glial inflammatory response (LaDu et al. 2000; LaDu et al. 2001; Laskowitz et al. 2001), but this is the first study to define specific LDL receptor family signaling pathways in microglial cells. These receptors interact with various cytoplasmic adaptor proteins that mediate receptor signaling (Gotthardt et al. 2000; Trommsdorff et al. 1998). The cytoplasmic tails contain NPxY...
sequences, which are recognized by various adaptor proteins that mediate intracellular signaling cascades. Of particular relevance to this study, the cytoplasmic domain of LRP1 binds JNK-interacting proteins (JIP-1 and JIP-2) (Stockinger et al. 2000). JIPs have been identified as both inhibitors and activators of the JNK signal transduction pathway (Dickens et al. 1997; Mooney and Whitmarsh 2004; Whitmarsh et al. 2001; Willoughby et al. 2003). In neurons, we found that the decrease in JNK activation by apoE receptors depends on release of the cytoplasmic domain from the cell membrane by γ-secretase (Hoe et al. 2005). Therefore, we propose that a similar mechanism may be employed in microglia. Both LRP1 and VLDLR have been found in microglia (Christie et al. 1996; Moestrup et al. 1992; Rebeck et al. 1993; Swanson et al. 1988) and we have identified them in primary glia by Western blot analysis (Supplemental Fig. 1B). Because a low concentration of RAP was used for these studies, we speculate that either LRP1 or VLDLR mediated the signaling effects of EP rather than the LDLR receptor, which is inhibited only by a high concentration of RAP (LaDu et al. 2000). However, exactly which of these receptors affect which kinase pathways is currently unknown.

In this work, we demonstrated that the LDL receptor family has immunomodulatory signaling properties based on studies of EP overcoming the LPS-induced inflammatory response in BV2 cells and primary microglia. We found that EP decreased JNK activation and thereby suppressed the LPS-induced JNK activation. Inhibition of JNK proved to be essential to overcome LPS-induced increase in NO and decrease in apoE production. EP also induced an increase in ERK activation, but
inhibition of ERK did not overcome the microglial inflammatory response. These studies provide new insight into the molecular mechanisms of LDL receptor family signaling in microglia and changes in apoE expression with microglial inflammation.
**Figure 1:** Activation of microglia by LPS increased NO and decreased apoE.

BV2 microglia and primary microglia were treated with 100 ng/ml LPS. **A.** In BV2 cells, LPS promoted nitrite accumulation in the conditioned media at 24 hrs and 48 hrs.
B. BV2 cell lysates were analyzed by Western blotting with antibodies to iNOS and β-actin (as a control). A representative blot shows that LPS increased iNOS levels (n = 4). C. BV2 cell lysates were analyzed for apoE and β-actin and conditioned media was analyzed for apoE. A representative blot shows that treatment of cells with LPS for 24 hrs reduced apoE in both cell lysates and media. D. Quantification of Western blots showed that apoE in cell lysate and media of BV2 cells was significantly decreased at 12 hrs, 24 hrs, and 48 hrs. The data were quantified as percent of control (One-Way ANOVA using Newman-Keuls Multiple Comparison Test for posthoc analysis, mean ± SEM; *P<0.05; **P<0.01 compared with corresponding control cultures; n = 6).

E. Primary microglia treated with LPS for 24 hrs accumulated a significant amount of nitrite in the conditioned media (Student’s T-Test, mean ± SEM; ***P<0.001; n=4). F. Cell lysates from primary microglia were analyzed by Western blotting with antibodies to iNOS and β-actin (as a control). A representative blot shows that LPS increased iNOS levels (n=3). G. Representative Western blots of primary microglia cell lysate analyzed for apoE and β-actin and conditioned media analyzed for apoE is shown. Microglia treated with LPS for 24 hrs showed reduced apoE in both cell lysates and media. H. Quantification of Western blot (Panel G) showed that apoE in cell lysate and media of primary microglia was significantly decreased at 24 hrs (Student’s T-Test, mean ± SEM; *P<0.05; ***P<0.001 compared with untreated cultures; n = 4).
Figure 2: ApoE peptide attenuated LPS induced NO increase and apoE decrease.

A. BV2 cells were treated with 100 ng/ml LPS and nitrite was measured in the conditioned media at 24 hrs. LPS induced nitrite accumulation and EP showed dose-dependent attenuation of LPS-induced nitrite production (n = 6). B. Cells were treated as in Panel A with 5 µM EP and LPS. EP attenuated LPS-induced nitrite production and 50 nM RAP prevented the effect of EP, while RAP alone had no effect (One-Way ANOVA using Newman-Keuls Multiple Comparison Test for posthoc analysis, mean
± SEM; **P<0.01 compared with corresponding control cultures; #P<0.05; ##P<0.01 compared with indicated cultures; n = 6).  

C. BV2 cell lysates were analyzed by Western blot analysis for apoE and β-actin. A representative blot is shown. LPS decreased apoE levels and 1 µM EP prevented that decrease, while EP alone had no effect on apoE levels. 50 nM RAP alone had no effect, but RAP prevented EP from reversing the effects of LPS. β-actin levels were the same across treatment conditions.

D. Western blot data were quantified as percent of control (One-Way ANOVA using Newman-Keuls Multiple Comparison Test for posthoc analysis, mean ± SEM; *P<0.05; **P<0.01; ***P<0.001 compared with control cultures; ##P<0.01 compared with indicated cultures; n = 3).  

E. Primary microglia were treated with 100 ng/ml LPS and nitrite was measured in the conditioned media at 24 hrs. LPS induced nitrite accumulation and 5 µM EP showed attenuation of LPS-induced nitrite production. 50 nM RAP prevented the effect of EP, while RAP alone had no effect (One-Way ANOVA using Newman-Keuls Multiple Comparison Test for posthoc analysis, mean ± SEM; ***P<0.001 compared with corresponding control cultures; #P<0.05; ##P<0.01 compared with indicated cultures; n = 3).  

F. Cells lysates from primary microglia were analyzed by Western blot analysis for apoE and β-actin. LPS decreased apoE levels and 1 µM EP prevented that decrease. β-actin levels were the same across treatment conditions (n = 3).
Figure 3: ApoE peptide increased ERK activation and reduced JNK activation.

BV2 microglia were treated with 1 µM EP or 100 ng/ml LPS from 5 to 60 min. Cell lysates were analyzed by Western blotting with antibodies to phospho-ERK, total ERK, phospho-JNK, and total JNK. A. Cells treated with LPS showed a time-dependent increase in both phospho-ERK and phospho-JNK. Total levels of ERK and JNK protein remain unchanged. B. Cells treated with EP showed a time-dependent increase in phospho-ERK and decrease in phospho-JNK. Total levels of ERK and JNK protein remain unchanged. C. Western blot data were quantified as percent.
change of phospho-ERK (upper graph) and percent change of phospho-JNK (lower graph) comparing EP and LPS treatment. LPS significantly increased phospho-ERK and phospho-JNK by 30 min. EP significantly increased phospho-ERK and decreased phospho-JNK by 30 min (One-Way ANOVA using Newman-Keuls Multiple Comparison Test for posthoc analysis, mean ± SEM; *P < 0.05; **P < 0.01; ***P < 0.001; n = 3).
Figure 4: ApoE peptide signaling effects are LDL receptor family mediated. BV2 microglia were treated with various combinations of PBS (control), 1 µM EP, 100 ng/ml LPS, and 50 nM RAP for 1 hr. Cell lysates were analyzed by Western blotting with antibodies to phospho-ERK, total ERK, phospho-JNK, and total JNK. A. Cells
treated with EP and LPS showed increased phospho-ERK and reduced phospho-JNK compared to cells treated with LPS alone. Treatment of BV2 cells with RAP prevented EP-induced potentiation of phospho-ERK and EP-induced attenuation of phospho-JNK. Total levels of ERK and JNK protein were unchanged. B. Western blot data were quantified as percent of control for phospho-ERK and phospho-JNK (One-Way ANOVA using Newman-Keuls Multiple Comparison Test for posthoc analysis, mean ± SEM; ##P < 0.01 compared with indicated cultures; n = 4).
Figure 5: JNK modulates NO production and apoE levels. A. BV2 cells were treated with indicated doses of a JNK inhibitor (SP600125), ERK inhibitor (PD98059), or PI3K/AKT inhibitor (Wortmannin), and stimulated with 100 ng/ml LPS for 24 hrs. SP600125 treatment reduced nitrite production in a dose-dependent manner. PD98059
and Wortmannin did not effect LPS induced nitrite production (n = 3).  **B.** Cell lysates were analyzed by Western blotting with antibodies to iNOS and β-actin. A representative blot shows that SP600125 reduced iNOS levels in a dose-dependant manner (n = 3).  **C.** BV2 cells were treated with suboptimal doses of EP and SP600125. Each alone showed attenuation of LPS-induced nitrite accumulation; in combination, they showed significant additive effects (One-Way ANOVA using Newman-Keuls Multiple Comparison Test for posthoc analysis, mean ± SEM; ***P < 0.001; **P < 0.01 compared with control cultures. ###P<0.001 compared with indicated cultures; n = 6).  **D.** Cell lysates of BV2 cells treated with suboptimal doses of EP and SP600125 were analyzed by Western blotting antibodies to iNOS and β-actin. Combined EP and SP600125 treatment reduced of LPS-induced iNOS accumulation (n = 3).  **E.** BV2 cells were treated with LPS and/or kinase inhibitors (i) and cell lysates were analyzed for apoE and β-actin expression. A representative blot shows that SP600125 increased apoE levels, but neither other inhibitor had any effects.  **F.** Western blot data were quantified as percent of control for apoE in the cell lysate. Cells treated with 1 µM SP600125 and LPS showed an increase in lysate apoE compared to cells treated with LPS alone, whereas cells treated with 10 µM PD98059 or 0.05 µM Wortmannin and LPS did not show an increase in lysate apoE compared to cells treated with LPS alone (One-Way ANOVA using Newman-Keuls Multiple Comparison Test for posthoc analysis, mean ± SEM; ***P < 0.001 compared with corresponding control cultures; ###P < 0.001 compared with indicated cultures; n = 3).
Figure 6: LDL receptor immunomodulatory signaling in microglia. LPS binds TLR4 receptors on the cell surface and signals an increase in phospho-JNK, leading to an increase in iNOS synthesis, an accumulation of NO extracellularly, and an independent decrease of intracellular apoE. ApoE binds LDL receptors on the cell surface and signals an increase in ERK activation and decrease in JNK activation. The decrease in JNK activation suppresses iNOS synthesis, reduces NO accumulation extracellularly, and suppresses a reduction of intracellular apoE.
A. BRIEF OVERVIEW

In our previous study, we demonstrated activation of member of the LDL receptor family regulates glial inflammation by modulating MAPK signaling pathways (Pocivavsek et al. 2009). We used an apoE mimetic peptide to activate these receptors and showed that the anti-inflammatory effects required reduction of MAPK family member c-Jun N-terminal kinase (JNK) activation (Pocivavsek et al. 2009).

The aims of our current study were to determine which of the LDL receptor family members expressed in microglia affected the JNK signaling pathway. We hypothesized that apoE binding to LRP1, whose cytoplasmic domain interacts with JNK-interacting proteins (JIPs) (Gotthardt et al. 2000), modulates JNK activation. LRP1 undergoes proteolytic processing that leads to release of the cytoplasmic tail (May et al. 2002). We have previously observed that ligand binding to the LDL receptor family can induce receptor proteolysis (Hoe and Rebeck 2005), release of cytoplasmic tail and adaptor proteins that may mediate intracellular signaling (Rebeck et al. 2006). To investigate whether LRP1 mediates the microglial inflammatory response, we have now used a mouse model where LRP1 was deleted in microglia and other cells of myeloid lineage. We monitored microglial activation by LPS through measures of nitric oxide (NO) accumulation and an increase in JNK activation. We used an apoE mimetic peptide (EP), consisting of a tandem repeat of the nine amino
acid receptor-binding domain to induce activation of LRP1. EP binds LDL receptor family members and activates intracellular signaling processes in microglia (Pocivavsek et al. 2009). Expression of LRP1 proved to be essential for EP to inhibit LPS-induced microglial inflammatory responses.

B. MATERIALS AND METHODS

Mice: Mice deficient in myeloid cell type-specific LRP were made using loxP/Cre-mediated recombination (Hu et al. 2006b); the mice were on an LDLR-deficient background as described previously (Lillis et al. 2008a). Briefly, as shown in Fig. 7, F1 generation mice were generated by breeding LRP-floxed mice on an LDLR-deficient background with LysMCre knock-in mice expressing Cre under the control of endogenous lysozyme M promoter to generate LRP flox+/+ Cre+/- LDLR+/+ mice. These mice were crossed with LRP-floxed mice on an LDLR-deficient background to generate two F2 generations: LRP flox+/+ Cre+/- LDLR-/- and LRP flox+/+ Cre-/- LDLR-/- mice. This generation of mice was crossed with one another yielding half of the siblings that carry no copies of Cre and thus express LRP normally (termed ‘wild-type’). The other half of the siblings carried one copy of Cre recombinase under the lysosome M promoter and thus generated deletion of LRP in myeloid cell types (termed ‘LRP -/-’).

Primary Microglial Cell Culture: Microglial cells were prepared from postnatal day 1 wild-type and LRP -/- mice as previously described (Pocivavsek et al. 2009). Briefly, cerebral cortices were carefully removed from pups, stripped of meninges and
homogenized in minimum essential media (MEM, Invitrogen) supplemented with 5% horse serum (Invitrogen), 5% fetal bovine serum (FBS), 1% L-glutamine (Invitrogen), 1% sodium pyruvate (Invitrogen), 1% Pen/Strep (Invitrogen), and 1% Fungizone (Invitrogen). The homogenized cells were centrifuged at 2000 rpm for 5 min, resuspended in fresh media and plated into poly-D-lysine (Sigma, St Louis, MO) coated T75 flasks. Mixed glial cultures were grown to confluency over 3 weeks and microglia were harvested by shaking the flasks at 100 rpm for 1 h at 37°C. The microglia-enriched media was collected and cells were pelleted by centrifugation (2000 rpm, 5 min). For immunocytochemistry microglia were resuspended in MEM supplemented with 10% FBS, 1% L-glutamine, 1% sodium pyruvate, 1% Pen/Strep, and 1% Fungizone and plated at a density of 1 x 10^5 cells/ml on poly-D-lysine-coated glass coverslips. Microglial cell purity was confirmed to be >95% using immunofluorescence for OX42 or Iba1 (microglial marker, Serotec, Raleigh, NC) and DAPI (Vector Laboratories, Burlingame, CA) nuclear counterstain (Supplemental Fig. 1). For biochemical experiments, cells were plated in 24-well plates at 12.5 X 10^4 cells / well, grown overnight in supplemented media and then the medium was replaced with serum-free Opti-MEM containing either control PBS or experimental agents.

**Antibodies:** Phosphorylation site-specific antibody against phospho-JNK (Thr183/Tyr185) (Cell Signaling Technologies, Beverly, MA) was used. Phospho-JNK antibody detected levels of p46 (JNK1) and p54 (JNK2 and JNK3) kinases when phosphorylated. Total JNK antibody (Cell Signaling Technologies) detected total levels of JNK proteins, including JNK proteins p46 and p54. Rabbit polyclonal
antibody was used to detect iNOS (BD Biosciences Pharmigen, San Diego, CA). From the same blots, β-actin (Abcam) was detected by monoclonal antibody to ensure equal protein levels in each lane. To detect LRP1, we used mouse monoclonal 5A6 that recognized the 85 kDa fragment of LRP1 (Misra et al. 1999). We also used a polyclonal antibody to detect the heavy chain of LRP1 (Lillis et al. 2008a). LRP1 was detected with anti-LRP Rb2629 (10ug/ml) by immunostaining (Lillis et al. 2008a).

**Immunocytochemistry:** Primary microglia on glass coverslips were fixed with 4% formaldehyde and 5% sucrose in PBS for 20 min at room temperature (RT), then washed 3 times with PBS. Fixed cells on coverslips were permeabilized with 0.4% Triton for 5 min at RT. Coverslips were blocked with 3% donkey serum for 1 hr and stained with anti-LRP Rb2629 overnight at 4°C. The coverslips were washed in PBS and stained with donkey anti-rabbit/Alexa488 and Phalloidin/Alexa468. Coverslips were mounted on glass slides with FluoroSave (Calbiochem).

**Chemicals:** The apoE-derived peptide_{141-149} (EP), consisting of a duplicated sequence of apoE amino acids 141 through 149, was synthesized by Johns Hopkins University of Medicine (Biosynthesis and Sequencing Facility, Baltimore, MD) (Hoe et al. 2005; Pocivavsek et al. 2009). Specific JNK inhibitor SP600125 was purchased from Invitrogen. Phosphatase inhibitor cocktails (Sigma) and protease inhibitor (Sigma) were used in cell lysis buffer. LPS was purchased from Calbiochem.

**Western Blot Analyses:** For analysis of cell associated proteins, cells were harvested in ice-cold lysis buffer containing phosphatase inhibitor cocktails and protease inhibitors. Proteins were separated under denatured and reduced conditions using Tris-
glycine SDS-polyacrylamide gel electrophoresis (Biorad, Hercules, CA). However, when detecting LRP, proteins were denatured but not reduced. Separated proteins were detected on PVDF membranes incubated with primary antibodies. Immunoreactivity was detected using horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) visualized by enhanced chemiluminescence detection film. Band density was determined using Quantity One 1-D analysis software (Biorad).

**Nitrite Quantification:** The production of NO was assessed as the accumulation of nitrite from the spontaneous oxidation of NO in serum-free cell conditioned media after 24 h. Accumulation of nitrite was quantified using a colorimetric reaction with Griess reagent (Invitrogen). Absorbance was measured at 570nm by spectrophotometry.

**Statistical Analysis:** All experiments were repeated a minimum of three times. The data were analyzed using Graphpad Prism 4 software, with either Student’s T-Test or ANOVA, using Newman-Keuls Multiple Comparison Test for posthoc analysis. Significance was determined at a P value of <0.05.

### C. RESULTS

**LRP1 deletion in primary microglia**

Recent studies with LRP floxed and LysMCre crossed mice have shown deletion of LRP1 in macrophages and microglia (Lillis et al. 2008a; Zhang et al. 2009). Our work with these mice showed successful LRP1 deletion in primary microglial
cultures by Western blot analysis (Fig. 8A) and immunocytochemistry (Fig. 8B). LRP1 expression was analyzed with two antibodies against LRP1, a mouse monoclonal that recognized the 85 kDa fragment of LRP1 and goat polyclonal that recognized full length LRP1. Both analyses revealed strong LRP1 expression in cell lysates from wild-type primary microglia and faint LRP1 expression in LRP -/- microglia (Fig. 8A). Western blot analyses of protein from cultures revealed that LRP1 levels in LRP -/- microglia remained at about 20% of wild-type microglia.

Primary microglial cells were immunostained with polyclonal antibody against LRP. Robust LRP1 expression was detected in wild-type microglia, with a significant reduction of LRP1 in LRP -/- microglia (Fig. 8B). Expression of LRP1 was detected in < 30% of LRP -/- microglia.

**Activation of Microglial Increases NO Production in a JNK-Dependent Manner**

Treatment of microglia with LPS induces intracellular signaling cascades, synthesis of inducible nitric oxide synthase (iNOS), and release of NO (Corradin et al. 1993). For our experiments, wild-type and LRP -/- microglia were treated with 100 ng/ml LPS in serum-free media for 24 h. LPS caused significant and similar increases in NO from both wild-type and LRP -/- microglia (Fig 9A). Wild-type cells treated with LPS accumulated 6.4 ± 0.5 μM nitrite compared with 1.4 ± 0.3 μM nitrite from untreated cells. LRP -/- cells treated with LPS accumulated 7.2 ± 0.6 μM nitrite, compared with 1.6 ± 0.6 μM nitrite from untreated cells. Expression of iNOS was undetected in untreated microglia, but easily found in extracts from wild-type or LRP -
cells treated with LPS for 24 h (Fig. 9B). Total protein levels of β-actin remained unchanged (Fig. 9B).

Previously, we showed that inhibition of JNK with SP600125 prevented LPS-induced NO accumulation in wild-type microglia (Pocivavsek et al. 2009). Next, we tested whether both wild-type and LRP-/- microglia respond to LPS treatment by activating JNK. As above, treatment of wild-type cells and LRP-/- microglia with LPS significantly increased nitrite accumulation over control treated cells (Fig. 9C). The JNK inhibitor SP600125 significantly reduced nitrite accumulation in a dose-dependent manner. With 10µM SP600125 treatment, levels of nitrite production were reduced to control levels in wild-type cells and slightly lower in LRP-/- cells (Fig. 9C).

**EP Attenuates LPS Induced NO Production in LRP Expressing Microglia Only**

Previously we have used EP, consisting of a tandem repeat of the receptor-binding domain of apoE, to demonstrate that LDL receptor family members can attenuate LPS-induced nitrite accumulation in microglia (Pocivavsek et al. 2009). However, the specific receptor through which EP mediated its anti-inflammatory properties was unknown. Here, we tested whether LRP1 mediates these effects. For these studies, EP treatment was performed at the same time as LPS stimulation. Wild-type and LRP-/- microglia were treated with two doses of EP, 1µM and 5uM. Wild-type microglia stimulated with LPS accumulated 8 ± 1 µM nitrite. When treated with 1µM EP and LPS, wild-type cultures accumulated 6.5 ± 1 µM nitrite, a 22 % decrease
compared to wild-type cells treated with LPS alone (data not shown). Cells treated with 5 µM EP and LPS accumulated 4 ± 0.3 µM nitrite, a significant 52% decrease (p < 0.05) compared with cells treated with LPS alone (Fig. 10A). This magnitude of an effect of EP was consistent with our previous studies (Pocivavsek et al. 2009).

LRP -/- primary microglia stimulated with LPS accumulated 9 ± 1 µM nitrite at 24 h. Cultures of LRP -/- microglia stimulated with 1µM EP and LPS accumulated 8.1 ± 1µM nitrite, a non-significant 14% decrease compared to LRP -/- microglia treated with LPS alone (data not shown). Cells treated with 5µM EP and LPS accumulated 6.6 ± 1µM nitrite, a non-significant 30 % decrease compared to cells treated with LPS alone (Fig. 10A). Further, when analyzing the difference between nitrite accumulation in wild-type and LRP -/- cultures treated with 5µM EP and LPS, we found that wild-type cultures had significantly less nitrite (4 ± 0.3 µM) accumulation compared to LRP -/- cultures (6.6 ± 1µM nitrite) (Fig. 10A).

Expression of iNOS was readily detected by Western blot analysis of primary microglia stimulated with LPS in both cell types. Wild-type microglia treated with EP and LPS showed decreased expression of iNOS when compared to cultures treated with LPS alone (Fig. 10B). Quantification of Western blot analysis revealed that expression of iNOS was reduced by 78 ± 6 % in wild-type microglia treated with EP and LPS compared to LPS alone (Fig. 10C). LRP -/- microglia treated with EP and LPS showed less reduced iNOS (39 ± 4 % reduction) when compared to cultures stimulated with LPS alone (Fig. 10B & C). Further analysis of iNOS expression
revealed that wild-type microglia treated with EP and LPS had significantly less iNOS expression than LRP -/- microglia treated with EP and LPS (Fig. 10C).

Because we observed a slight reduction in NO production in the LRP -/- microglia treated with EP, we tested whether another apoE receptor, VLDLR, was also partially involved in mediating the inhibitory effects of EP on microglia activation. To block EP from binding VLDLR, we used an anti-VLDLR cocktail designed by combining titers from mice used to generate monoclonal antibodies 5F3, 1H5, and 1H10, as previously described (Ruiz et al. 2005). For these experiments, wild-type microglia treated with LPS accumulated a significant increase in nitrite (22 ± 2 µM) when compared to control cultures (3 ± 0.4 µM). Similar to our previous experiments in Fig. 10A, wild-type cultures treated with EP and LPS showed a significant 54% reduction in nitrite (10 ± 0.4 µM) when compared to cultures stimulated with LPS alone (Fig. 10D). Cultures simultaneously treated with anti-VLDLR, EP and LPS accumulated 10 ± 0.2 µM nitrite, not significantly different from cultures treated with EP and LPS alone. Treatment with anti-VLDLR and LPS resulted in 17 ± 0.1 µM nitrite and treatment with cold IgG resulted in 21 ± 0.1 µM nitrite, both not significantly different from wild-type cultures treated with LPS alone (Fig. 10D).

We observed similar effects in LRP -/- microglia. LRP -/- microglia treated with LPS accumulated an increase in nitrite (32 ± 2 µM) over control cultures (3 ± 0.1 µM), as we previously described in Fig 9. Further, treatment of LRP -/- microglia with EP and LPS reduced nitrite accumulation by 34% (21 ± 2µM), similar
to what we previously described in Fig. 10A. We used anti-VLDLR in combination with EP and LPS in LPR -/- microglia and we observed that nitrite accumulation was not altered when VLDLR was blocked. LRP -/- cultures treated with anti-VLDLR, EP and LPS accumulated 20 ± 1\( \mu \)M nitrite, similar to cultures treated with EP and LPS (Fig. 10D). Cultures of LRP -/- microglia treated with anti-VLDLR accumulated 32 ± 1 \( \mu \)M nitrite and cultures treated with cold IgG accumulated 39 ± 1 \( \mu \)M nitrite, both similar to LRP -/- microglia stimulated with LPS alone (32 ± 2 \( \mu \)M nitrite) (Fig. 10D). Taken together, our data support the idea that LRP1 mediates the inhibitory effects of EP on NO production and that VLDLR does not contribute to this effect. We suggest that the slight abrogation of the LPS-effect by EP in LRP -/- microglia may be due to the 20 % of cells that are expressing LRP in the knockout cultures (as shown in Fig. 8).

**EP reduces JNK activation only in LRP expressing microglia**

We have previously shown that EP treatment decreased JNK through the LDL receptor family and counteracted LPS-induced inflammatory responses through this modulation (Pocivavsek et al. 2009). The data in Fig. 10 show that EP significantly counteracts LPS-induced inflammatory response in wild-type microglia, but not LRP -/- microglia. We next asked whether EP treatment modulated JNK activation via LRP1. JNK phosphorylation was examined 1 h after treatments. Wild-type microglia treated with EP had decreased phospho-JNK levels, whereas levels of total JNK remained unchanged (Fig. 11A) when compared to control cultures. EP-treated wild-type microglia showed a non-significant 22 % decrease in phospho-JNK levels (Fig.
Treatment of wild-type microglia with LPS for 1 h significantly increased phospho-JNK expression and total levels of JNK remained unchanged (Fig. 11A). Wild-type microglia treated with LPS had 591 ± 82% of control levels of phospho-JNK. Simultaneous treatment of wild-type microglia with EP and LPS induced a significant 48% decrease in phospho-JNK (307 ± 50% of control) levels when compared with cultures treated with LPS alone (Fig. 11B). To test whether LRP1 was involved in the effects of EP on JNK signaling, we tested LRP-/- microglia. As with wild-type cells, treatment of LRP-/- cultures with EP did not reduce basal phospho-JNK expression when compared to control cultures (Fig. 11A). LRP-/- microglia stimulated with LPS had 555 ± 98% of control levels of phospho-JNK, a significant increase that was similar to the increase observed in wild-type microglia stimulated with LPS (Fig. 11B). In contrast to wild-type microglia, LRP-/- microglia simultaneously treated with EP and LPS did not show a reduction in phospho-JNK (567 ± 105% of control) levels when compared with cultures treated with LPS alone (Fig. 11B). These data indicated that EP attenuates LPS-induced JNK activation only in LRP1 expressing microglia.

D. DISCUSSION

This study shows that activation of LRP1 can modulate microglial inflammation by reducing phosphorylation of JNK. We confirmed that LRP1 is expressed in microglia (Pocivavsek et al. 2009) and sought to activate LRP1 by
applying an apoE mimetic peptide, EP, which attenuated LPS-induced microglial inflammation. In wild-type microglia, LPS activation of JNK increased NO production and EP treatment decreased JNK and counteracted LPS-induced inflammation. In microglia lacking LRP1, while LPS activation of JNK also increased NO production, EP treatment did not decrease JNK and its inhibition of NO was significantly attenuated. Taken together, these data suggest that LRP1 regulates the microglial inflammatory response by suppressing activation of JNK.

While members of the LDL receptor family have previously been implicated in modulating the glial inflammatory response (LaDu et al. 2000; LaDu et al. 2001; Laskowitz et al. 2001; Pocivavsek et al. 2009), this is the first study to define an LRP1-dependent immunomodulatory cascade in microglia. LRP1 contains a single transmembrane domain and a cytoplasmic domain consisting of 100 amino acids, including two NPxY motifs that are tyrosine phosphorylated (Lillis et al. 2008b). This cytoplasmic tail interacts with various adaptor protein molecules implicated in cell signaling events including JNK-interacting proteins, JIP-1 and JIP-2 (Gotthardt et al. 2000). JIPs have been implicated as inhibitors and activators of the JNK signaling pathway (Dickens et al. 1997; Mooney and Whitmarsh 2004; Whitmarsh et al. 2001; Willoughby et al. 2003). We postulate that upon LRP1 activation, the C-terminal fragment (CTF) of LRP1 is released from the plasma membrane, carrying with it JIP proteins. This LRP1-CTF-JIP complex could then travel to other subcellular domains and modulate the activation of JNK at those sites. We have reported that ligand-induced proteolytic cleavage of LDL receptor family members in neurons modulates
JNK activation (Hoe et al. 2005; Hoe et al. 2006), and we propose a similar mechanism may be occurring in microglia.

LRP1 undergoes proteolytic processing by γ-secretase, an intramembrane protease that processes many substrates (Parks and Curtis 2007). Upon γ-secretase cleavage, the intracellular domain of LRP1 (LRP1-ICD) is released into the cytoplasm (May et al. 2002). A recent study showed that proteolytic processing of LRP1 by γ-secretase and the subsequent translocation of LRP1-ICD into the nucleus regulates inflammation in peritoneal macrophages (Zurhove et al. 2008). These findings support our hypothesis that proteolytic processing of LRP1 mediates its anti-inflammatory effects.

In the CNS, LRP1 is expressed in neurons, astrocytes, and microglia. In neurons, LRP1 has been implicated in modulating Alzheimer’s disease pathology by altering the trafficking of amyloid precursor protein (APP) and production of the toxic Aβ peptide (Pietrzik et al. 2002; Ulery et al. 2000; Ulery and Strickland 2000). Studies have also implicated LRP1 in clearing Aβ by receptor-mediated endocytosis (Deane et al. 2004; Lillis et al. 2008b). Apart from its effects on AD pathological processes, neuronal LRP1 has been implicated in mediating long-term potentiation and calcium influx through the NMDA receptor (Bacskai et al. 2000; Herz 2001). LRP1 contributes to blood-brain barrier (BBB) permeability through effects on perivascular astrocytes (Yepes et al. 2003). Recently, it was found that tissue-type plasminogen activator (tPA) regulates the permeability of the BBB via an interaction with LRP1 (Polavarapu et al. 2007; Yepes et al. 2003; Zhang et al. 2007). tPA is a serine
protease and activator of plasminogen (Bugge et al. 1996) that is used clinically to treat acute ischemic stroke. Increasing evidence shows that tPA has deleterious effects over time and most recently the interaction between tPA and LRP1 has been characterized as pro-inflammatory (Hu et al. 2006a; Wang et al. 2003; Yepes et al. 2003; Zhang et al. 2009; Zhang et al. 2007). While it was demonstrated that tPA mediates deleterious effects on the ischemic brain via LRP1-dependent activation of microglia (Zhang et al. 2009), the mechanism by which tPA induces LRP1 mediated microglial activation remains to be elucidated. However, our work suggests that LRP1 activation by an apoE mimetic reduces JNK activation and thus downregulates the microglial inflammatory response induced by LPS stimulation. The contrasting roles of LRP1 ligands, apoE and tPA, suggests unique and specific ligand-induced physiological functions of LRP1 in microglia.

We propose that activation of LRP1 by apoE regulates immunomodulatory signaling cascades in microglia. Immunomodulatory properties of LRP1 may have implications for AD. Astrocytes and microglia surround amyloid deposits in AD (Ard et al. 1996; Paresce et al. 1996; Schenk et al. 1999) and both cell types express LRP1 (Christie et al. 1996; Pocivavsek et al. 2009; Rebeck et al. 1993). Further, LRP1 associates with amyloid plaques in AD and its expression is upregulated in glial cells (Arelin et al. 2002). The glial activation seen in AD brains may demonstrate a failure of LRP1 immunomodulatory functions in the presence of chronic insults by Aβ oligomers (LaDu et al. 2000; LaDu et al. 2001). We hypothesize that LRP1 expressed on astrocytes may have similar immunomodulatory properties that are impaired when
astrocytes surround amyloid plaques. Development of astrocyte-specific LRP1 knock-out mice would help elucidate the role of LRP1 on astrocytes specifically.

Our work provides insight into LRP1 signaling and shows that LRP1 activation modulates the inflammatory response of microglia. In our studies, microglia were activated with LPS treatment, which induced an increase in JNK activation and NO production. We found expression of LRP in microglia was essential for EP to reduce JNK activation and suppress LPS-induced JNK activation. While inhibition of JNK with a synthetic inhibitor proved to overcome LPS-induced increase in NO in both wild-type and LRP −/− microglia, EP suppressed JNK activation only in LRP expressing microglia. We suggest that LRP1 on microglia and astrocytes represents a target for anti-inflammatory therapies in AD and other CNS diseases.
Figure 7: Breeding schematic to generate LRP1 knockout in microglia. LRP-floxed mice were bred with LysMCre mice to generate an F1 generation of LRP flox +/- Cre +/- LDLR +/- mice. This F1 generation was breed with LRP-floxed mice to generate two F2 generations that were then crossed with one another to yield 50% LPR-expressing (termed ‘wild-type’) and 50% LRP-deficient mice (termed ‘LRP-/-’).
Figure 8: LRP1 knockout in primary microglia. A) Cell lysates from wild-type (wt) and LRP -/- (ko) microglia were analyzed by Western blotting for LRP expression. The left panel shows a representative blot probed with 5A6, recognizing a 85 kDa band for LRP. The right panel shows a blot probed with goat α-LRP, recognizing full length LRP. B) Primary microglia from wild-type (WT) and LRP -/-
cultures were immunostained with anti-LRP Rb2629 (10ug/ml). The primary antibody was detected with donkey anti-rabbit / Alexa488 and cell morphology was detected with Phalloidin / Alexa 469.
Figure 9: Stimulation of microglia with LPS increased NO through JNK activation. A) In wild-type and LRP -/- microglia, LPS promoted nitrite accumulation.
in the conditioned media at 24 h (Student’s T-Test, mean ± SEM; ***P < 0.001; n=6).

**B)** Cell lysates from wild-type and LRP -/- microglia were analyzed by Western
bloTTing with antibodies to iNOS and β-actin (as a control). A representative blot
shows that LPS increased iNOS levels in both cell types (n=6). **C)** Wild-type and LRP
-/- microglia were treated with LPS and SP600125. In a dose-dependent manner,
SP600125 treatment reduced nitrite production in both wild-type and LRP -/- microglia
(One-Way ANOVA using Newman-Keuls Multiple Comparison Test for posthoc
analysis, mean ± SEM; **P < 0.01 compared to control cultures; # P < 0.05, ## P <
0.01 compared to corresponding cultures; n=3).
Figure 10: ApoE peptide attenuated LPS-induced NO production in LRP1 expressing microglia only. A) Wild-type and LRP -/- microglia were treated with 100
ng/mL LPS and nitrite was measured in the conditioned media at 24 h. LPS induced nitrite accumulation in both cell types and EP showed attenuation of LPS-induced nitrite production only in wild-type cultures (One-Way ANOVA using Newman-Keuls Multiple Comparison Test for posthoc analysis, mean ± SEM; *** P < 0.001 compared to corresponding control cultures; ## P < 0.01, ### P < 0.001 compared with indicated cultures; n = 6). B) Cell lysates from wild-type and LRP -/- cultures were analyzed by Western blotting for iNOS and β-actin. Representative blots show that EP and LPS treatment reduced iNOS expression compared to LPS stimulation alone in wild-type stimulated cultures but not LRP -/- cultures. C) Western blot data were quantified as percent of control (One-Way ANOVA using Newman-Keuls Multiple Comparison Test for posthoc analysis, mean ± SEM; * P < 0.05, ** P < 0.01 compared with LPS stimulated cultures; # < 0.05 compared with indicated cultures; n = 6). D) Wild-type (left panel) and LRP -/- (right panel) cultures were treated with LPS and nitrite was measured in the conditioned media at 24 h. LPS induced nitrite accumulation in wild-type and LRP -/- cultures. EP and LPS treatment attenuated LPS-induced nitrite accumulation significantly in wild-type cultures and treatment with 500 nM VLDLR (α-VLDLR) blocking antibody, EP and LPS did not prevent the effects of EP. Similarly in LRP -/- microglia, nitrite accumulation in cultures treated with VLDLR antibody cocktail to block VLDLR ligand binding did not significantly differ from cultures treated with EP and LPS (One-Way ANOVA using Newman-Keuls Multiple Comparison Test for posthoc analysis, mean ± SEM; ns means no significant; n = 4).
Figure 11: ApoE peptide signaling effects are LRP1 mediated. Wild-type and LRP -/- primary microglia were treated with PBS (c), 1 µM EP, 100 ng/mL LPS, and 1 µM EP with LPS for 1 h. Cell lysates were analyzed by Western blotting with antibodies to phospho-JNK and total JNK. A) Wild-type cells treated with EP showed decreased phospho-JNK compared with cells treated with control PBS. Treatment of wild-type cells with LPS increased phospho-JNK. Cells treated with EP and LPS showed reduced phospho-JNK compared to cells treated with LPS alone. LRP -/- cells treated with LPS showed increased phospho-JNK compared with cells treated with control
PBS. In LRP -/- cultures, EP and LPS treatment did not reduce phospho-JNK compared to cells treated with LPS alone. B) Western blot data were quantified as percent of control phospho-JNK (One-Way ANOVA using Newman-Keuls Multiple Comparison Test for posthoc analysis, mean ± SEM; *** P < 0.001 compared with control cultures; ## P < 0.01 compared with indicated cultures; n = 3).
Chapter IV: Inhibition of C-Jun N-Terminal Kinase Increases ApoE

*In Vitro* in Primary Glia and *In vivo* in Mouse Brain

A. BRIEF OVERVIEW

Activation of members of the LDL receptor family decreased JNK activation in isolated microglia and increased apoE levels (Pocivavsek et al. 2009). ApoE has anti-inflammatory properties in the CNS, as demonstrated in several *in vitro* and *in vivo* systems (Laskowitz et al. 1997; Laskowitz et al. 2000; Laskowitz et al. 1998; Laskowitz et al. 2007; Laskowitz et al. 2001; Lynch et al. 2001; Lynch et al. 2003; Lynch et al. 2005; Mace et al. 2007; Pocivavsek et al. 2009). Our *in vitro* studies suggested that JNK inhibition maybe an effective way to increase apoE protein *in vivo* and this increase in apoE could be anti-inflammatory.

Our current study aims to investigate the impact of JNK inhibition on apoE production in the brain. For these studies, we used two structurally and functionally different JNK inhibitors, JNK inhibitor I (L)-Form (L-JNK1) and SP600125. L-JNK1 is a small cell-permeable peptide that blocks the activation domain of JNK, preventing activation of the transcription factor c-Jun. SP600125 is also a cell-permeable small molecule that selectively inhibits all three JNK isoforms and prevents phosphorylation of downstream the JNK target c-Jun. We studied apoE expression in primary glia treated with JNK inhibitors and *in vivo* in mice injected with JNK inhibitor. We also used APOE promoter luciferase reporter constructs to define the importance of the
proximal APOE promoter for the effects of JNK. Our work showed that APOE transcription and apoE protein expression are increased with JNK inhibition.

B. MATERIALS AND METHODS

Primary Glial Culture: Primary mouse mixed glial cultures were prepared from postnatal day 1 Swiss-Webster mouse pups. Briefly, cerebral cortices were carefully removed from pups, stripped of meninges and homogenized in minimum essential media (MEM, Invitrogen) supplemented with 5% horse serum (Invitrogen), 5% FBS, 1% L-glutamine (Invitrogen), 1% sodium pyruvate (Invitrogen), 1% Pen/Strep (Invitrogen), and 1% Fungizone (Invitrogen). The homogenized cells were centrifuged at 2000 rpm for 5 min, resuspended and plated into poly-D-lysine (Sigma, St Louis, MO) coated T75 flasks. Mixed glial cultures were grown at 37°C under 5% CO₂. After 2 days, the media was removed and replaced with fresh complete media. Cultures were grown to confluency (approximately 14 days in culture) with media replenished every 3 days. For experiments, cells were plated in 24-well plates at 12.5 X 10⁴ cells/well. Plated cells were grown in supplemented media overnight and then the medium was replaced with serum-free Opti-MEM containing either control PBS or experimental agents.

Intrahippocampal Injections: Adult Swiss-Webster mice (30-40g, Taconic) were anesthetized with ketamine and xylazine (Sigma). Animals were placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). A single, 5 µl injection of control, 10 µl injection of control, 5 µl injection of 10mM SP600125
(11.01 µg), or 10 µl injection of 10mM SP600125 (22.02 µg) was delivered to the right hippocampus (coordinates from bregma: -1.46 posterior, -1.0 mm lateral, and -2.0 mm ventral). SP600125 was dissolved in 5 % DMSO and 50 % EtOH in PBS and, thus, the control injection was 5 % DMSO, 50 % EtOH in PBS. Solutions were delivered by a microinfusion pump at a constant flow of 0.5 µl/min. The incision was closed with surgical suture and animals were returned to individual cages.

**Tissue Preparation:** Animals were sacrificed 24 hrs after intrahippocampal injection. Mice were overdosed with ketamine and perfused transcardially with PBS. The brain was removed and the hippocampi and cortices were dissected bilaterally. Proteins from each brain area were extracted in RIPA buffer (50mM Tris-HCL, pH 8.0M NaCl, 0.1% Triton X-100) with phosphatase and protease inhibitors. Samples were sonicated for 10 sec, centrifuged at 4°C for 10 min at 14,000 rpm in a microcentrifuge, and the supernatant solution was collected.

**Western Blot Analyses:** For all blots, protein levels were detected using the Bradford protein assay (Biorad). 15-20µg protein was loaded into each well for all blots. For analysis of secreted molecules, conditioned media was collected from cultured cells. Cell associated proteins were harvested in ice-cold lysis buffer containing phosphatase inhibitor and protease inhibitor cocktails. Proteins were separated under denatured and reduced conditions using Tris-glycine SDS-polyacrylamide gel electrophoresis (Biorad, Hercules, CA). Separated proteins were detected on PVDF membranes incubated with primary antibodies. Immunoreactivity was detected using horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove,
PA) visualized by enhanced chemiluminescence and detected with film. Band density was determined using Quantity One 1-D analysis software (Biorad).

**Antibodies:** ApoE was detected by rabbit polyclonal antibody against rodent apoE (Abcam, Cambridge, MA). ABCA1 was detected by a mouse monoclonal antibody (Biorad). All blots were probed with a monoclonal β-actin (Abcam) antibody to ensure equal protein levels in each lane.

**Chemicals:** SP600125 was purchased from Invitrogen. The polypeptide JNK inhibitor, L-JNK1, was purchased from Calbiochem (San Diego, CA). Wortmannin and PD98059 were purchased from Sigma. LPS was purchased from Calbiochem.

**Plasmids:** Human APOE promoter pGL1052 (-1054 to +44 nucleotides from the transcription start site) and the corresponding pGL3 empty vector were generously provided by Dr. Debomoy Lahiri (Indiana University School of Medicine, Indianapolis, IN)(Du et al. 2005). Human APOE promoter fragments pGL623en (-623 to +86), pGL503en (-503 to +86), pGL503 (-503 to +86), pGL300 (-300 to +86) were generously provided by Dr. Theodore Mazzone (University of Illinois at Chicago, IL) (Yue et al. 2008). The pGL623en and pGL503en constructs included the 620-bp downstream enhancer (ME1) of the human APOE gene.

**Transient Transfection and Luciferase Activity:** BV2 microglia (Blasi et al. 1990; Pocivavsek et al. 2009) were plated at 3.75 x 10^4 cells / well in 24-well plates (500 µl/well). Plated cells were grown in Opti-MEM with 5% BS overnight and transfected with 0.1 µg of the APOE reporter construct and 0.01 µg of Renilla reporter construct with 0.6 µl Fugene6 according to manufacturer’s instructions (Roche). After 6 hrs, the
medium was replaced with serum-free Opti-MEM containing either control PBS or experimental agents and incubated overnight. After the treatments, cells were washed in cold PBS and lysed in 60 µl passive lysis buffer reagent from the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). 20 µl of cell lysate was plated into 96-well plate and the luminescence for Firefly and Renilla luciferases were determined using a luminometer (Tecan Ultra microplate reader, Lombardi Cancer Center, Georgetown University, Washington, DC). Measures of Renilla were used to ensure that there was minimal variation due to transfection differences. Each condition was performed with at least 3 sets of cells and measurements of each sample were performed in duplicate.

**Quantitative RT-PCR:** Total RNA was isolated from primary mixed glial cultures using Stratagene Absolutely RNA Miniprep Kit (Stratagene). First-strand cDNA was synthesized from 1 µg total RNA using Affinity Script QPCR cDNA Synthesis Kit according to manufacturer’s instructions. cDNA (1 µl) was amplified by real-time PCR using Power SYBR Green PCR Master Mix (Applied Biosystems). Samples were standardized to β-actin message amount. Synthetic oligonucleotides ApoE (5’-TCGGAAGGAGCTGACTGG-3’) and β-actin (5’-TGACAGGATGCAGAAGGAGA-3’) were used. Samples were incubated at 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 sec, 58°C for 1 min and 72°C for 30 sec. In every case, cycle threshold taken for quantitation was in the linear portion of the amplification range. Each individual sample was analyzed in triplicate and RNA levels are reported as fold change compared with control. Reaction product purity was confirmed by examining
the melting curves for a single peak. Analysis of real-time amplification data was done on SDS 2.3 (Applied Biosystems) and relative quantities were calculated using RQ Manager software (Applied Biosystems).

**Nitrite Quantification:** The production of NO from glial cultures was assessed as the accumulation of nitrite from the spontaneous oxidation of NO in serum-free cell conditioned media after 24 hrs using a colorimetric reaction with Griess reagent (Invitrogen). Absorbance was measured at 570nm by spectrophotometry.

**Statistical Analysis:** All experiments were repeated a minimum of three times. The data were analyzed using Graphpad Prism 4, performing either ANOVA with Newman-Keuls Multiple Comparison post-hoc test or Student’s t-test analysis with significance determined at a P value of <0.05.

C. **RESULTS**

**JNK-Dependent Effects of Glial Stimulation on NO Production**

We have previously shown that LPS induces intracellular activation of JNK, downstream synthesis of inducible nitric oxide synthase (iNOS), and release of NO in microglia (Pocivavsek et al. 2009). For the current experiments, mouse mixed glial cultures were treated with 100 ng/mL LPS in serum-free media. As expected, LPS treatment caused a significant increase NO after 24 hrs of LPS stimulation (Fig. 12A). Primary cultures treated with LPS accumulated 16 ± 1 μM nitrite, compared with 2 ± 0.5 μM nitrite from untreated cells. We used inhibitors to the MAPK pathway family members, JNK and ERK, and an inhibitor to phosphoinositol 3-kinase (PI3K)/protein
kinase B (Akt) pathway. Cells were treated with two doses of JNK inhibitor (SP600125), ERK inhibitor (PD98059), and PI3K/Akt inhibitor (Wortmannin) to test which signaling pathways mediated the NO response. The lowest dose used for each inhibitor blocked the phosphorylation of its respective kinase at 1 hr (data not shown). Mixed glia treated with LPS and SP600125 accumulated significantly less nitrite (13 ± 0.7 µM with 1 µM SP600126 and 5 ± 0.5 µM with 10 µM SP600125) in a dose-dependent manner when compared with cultures treated with LPS alone (16 ± 0.3 µM nitrite) (Fig. 12B). Cultures treated with PD98059 or Wortmannin showed no attenuation of the LPS-induced nitrite response (Fig. 12B). These findings are consistent with reports that JNK activation is required for NO production (Moon et al. 2007; Pawate and Bhat 2006; Pocivavsek et al. 2009). We next asked whether a different JNK inhibitor would also overcome LPS-induced nitrite accumulation. Primary mixed glial cultures were stimulated with LPS and either SP600125 or L-JNK1. Cells treated with LPS and SP600125 accumulated significantly less nitrite (3 ± 0.1 µM) than cells treated with LPS alone (12 ± 0.3 µM nitrite); cells treated with LPS and L-JNK1 also accumulated significantly less nitrite (5 ± 0.1µM) than cells treated with LPS alone (Fig. 12C). Taken together, these findings show that JNK activation, but not ERK or PI3K/Akt activation, is important for LPS-induced NO accumulation in glia.
Activation of glia decreases apoE production and secretion

Previous reports show that apoE is downregulated in microglia activated with LPS (Pocivavsek et al. 2009; Saura et al. 2003). We tested whether there were similar effects in primary mixed glial cultures since most apoE \textit{in vivo} is synthesized in astrocytes. Glial cultures were treated with LPS for 24 hrs. The relative quantity of apoE mRNA was measured by quantitative real-time PCR and normalized to β-actin mRNA expression. Cultures treated with LPS expressed 48% less APOE mRNA than control untreated cultures (Fig. 13A). LPS treatment also reduced endogenous apoE protein in cell lysates and conditioned media of primary glia (Fig. 13B). Total protein levels of β-actin remained unchanged. Quantification of Western blot analysis showed that apoE levels in mixed glial lysates and conditioned media significantly decreased with LPS stimulation when compared with apoE levels in untreated cells at 24 hrs (Fig. 13C). LPS reduced glial apoE levels by 58% in the cell lysates and 26% in conditioned media (Fig. 13C).

Inhibition of JNK increases ApoE production and secretion

We next asked whether inhibition of JNK alone increased apoE expression. Mixed glial cultures were treated with 10µM of the JNK inhibitor SP600125 for 24 hrs. APOE mRNA expression was quantified by quantitative real-time PCR. Cultures treated with SP600125 showed a 50% increase in APOE mRNA expression compared to control untreated mixed glial cultures (Fig. 14A). We also examined apoE protein levels and observed that primary glia treated with SP600125 showed increased apoE
compared to untreated cultures (levels of β-actin remained unchanged) (Fig. 14B). Quantification of Western blot analysis revealed that SP600125 treatment increased apoE protein levels in primary glia by 98% (Fig. 14C). Treatment of mixed glial cultures with another JNK inhibitor, L-JNK1, also significantly increased apoE compared to untreated cultures (Fig. 14B). Western blot analysis showed that L-JNK1 increased apoE in cell lysates by 72% (Fig. 14C). We also studied expression of ABCA1, a member of the ATP-binding cassette superfamily of transporters that transports cholesterol and phospholipids across the membrane (Oram and Heinecke 2005). Changes to ABCA1 levels have also been linked with changes to apoE levels in glia (Hirsch-Reinshagen et al. 2004). We hypothesized that with increased apoE expression we might also see an increase in ABCA1 expression. Indeed, we observed that inhibition of JNK with either SP600125 or L-JNK1 increased both apoE and ABCA1 (Fig. 14B).

**JNK inhibition increases proximal APOE gene promoter activity**

We next used the APOE promoter-luciferase reporter constructs shown in Fig. 15A (Du et al. 2005; Yue et al. 2008) to study the effects of JNK inhibition on APOE promoter activity in BV2 cells. The constructs contained an APOE 5’ promoter sequences of various lengths with or without the 620-bp ME1 enhancer element (drawn as a boxed ‘en’ in the figure). Total luciferase activity decreased with truncation of the APOE promoter (data not shown), consistent with previous studies of the proximal promoter (Du et al. 2005; Yue et al. 2008). The JNK inhibitor SP600125
significantly increased luciferase activity of pGL1052 (132 % over control treatment), the longest APOE promoter construct, but did not affect the activity of the empty pGL reporter (Fig. 15B). This result indicated that a JNK regulatory region is present on the APOE promoter. We then tested shorter APOE promoter constructs. SP600125 increased luciferase activity for all constructs, thus we concluded that the JNK regulatory region is still present even in the shorter constructs. To compare the magnitudes of increase by SP600125 on reporter constructs with different baseline expression, we examined changes in reporter expression relative to untreated control for each construct (Fig. 15B). We observed that SP600125 treatment increased apoE promoter activity in the smallest promoter construct examined, pGL300, by 126 % over control treatment (Fig. 15B). This result indicated that the regulatory elements on the apoE promoter necessary for the effects of SP600125 were contained within the -300 and +86 portion of the APOE gene.

**Inhibition of JNK increases apoE expression in vivo**

We have shown that inhibition of JNK increased APOE mRNA and protein expression and increased APOE promoter activity in mixed glial cultures (Figs. 14 and 15). We next tested whether similar effects were observed *in vivo*. We injected 5µl or 10µl SP600125 (10mM) or vehicle control (5 or 10 µl) into the right hippocampus of adult mice. After 24 hrs, the cortices and hippocampi were dissected and homogenized for Western blot analysis. Animals injected with either 5µl or 10µl SP600125 showed increased apoE expression in the ipsilateral hippocampus when compared to control
injected animals, while apoE expression in the cortex remained unchanged (Fig. 16A). Because expression appeared unchanged in the cortex, we focused our analysis on apoE levels only in the hippocampus. Levels of apoE expression were similar in the contralateral hippocampus from animals injected with vehicle or either 5 µl or 10 µl SP600125 (Fig. 16B). ApoE levels in the ipsilateral hippocampus increased in animals injected with 5 µl or 10 µl SP600125 when compared to vehicle injected animals (Fig. 16B). Mice injected with 5 µl SP600125 had 48 ± 14 % more apoE than control injected mice (P < 0.05), while mice injected with 10 µl SP600125 had a 150 ± 34 % increase in apoE (P < 0.001) (Fig. 16C). The contralateral hippocampus showed no significant differences in apoE between control, 5 µl or 10 µl SP600125 injection. We also looked at expression of ABCA1 and observed that ABCA1 increased with apoE in mice injected with 5 µl or 10 µl SP600125 (Fig. 16B). ABCA1 expression remained unchanged in the contralateral hippocampus.

In an independent experiment, we also injected 10µl SP600125 into the hippocampus of 3 mice and compared them with 3 vehicle injected mice. This experiment also revealed that SP600125 injection into the hippocampus of mice increased apoE expression significantly when compared to control injected mice. The increase in apoE expression in the ipsilateral hippocampus of SP600125 mice was 120 ± 27 % compared to the ipsilateral hippocampus of control injected mice (data not shown). In this experiment, we also observed a small increase in the cortex after SP600125 (30 ± 10 %) (data not shown). Thus, from two independent experiments, we
observed an increase in apoE levels with SP600125 in the mouse hippocampus, supporting the hypothesis that inhibition of JNK increases apoE expression \textit{in vivo}.

**D. DISCUSSION**

Our study shows that inhibition of JNK promotes expression of APOE. When we inhibited JNK, we showed that APOE expression increased in mixed glial cultures \textit{in vitro} and in mouse brains. We showed that JNK inhibition increased the activity of the proximal APOE promoter (-300/ +86), where an AP-1 binding site has been predicted (Maloney et al. 2007). Taken together, we developed a model of APOE gene regulation by c-Jun and AP-1 transcription factor activation (Fig. 17). We also demonstrated that an inflammatory response in glial cells stimulated with LPS reduced APOE mRNA and protein expression. The response to LPS was overcome by modulating JNK activation in glial cells. We suggest that LPS stimulation increases phospho-JNK, activating downstream c-Jun and promoting AP-1 to bind the APOE promoter. By binding the APOE promoter downstream of the TATAA sequence, AP-1 interferes with transcriptional start and reduces transcription of APOE mRNA. Our work shows that expression of APOE mRNA and apoE protein can be increased by inhibiting JNK.

The JNK family includes three isoforms, termed JNK1, JNK2, and JNK3, which are expressed in glial cells (Hidding et al. 2002). Upon activation, JNK2 and JNK3 move to the nucleus rapidly and exert activation of transcription factors. JNK1 remains constitutively present in the nucleus. All three JNK isoforms are inhibited by
the compounds used in this study, SP600125 and L-JNK1. JNK phosphorylation leads to the activation of c-Jun and AP-1, a transcription factor composed of heterodimers of family members, which includes Jun, Fos and ATF (Ip and Davis 1998). AP-1 proteins function in several ways including inducing transcription of some genes and suppression of others (Herdegen and Waetzig 2001). As inducers of transcription, AP-1 proteins bind to AP-1 consensus motifs (5’-TGAG/CTCA-3’) and orchestrate the start of transcription. AP-1 proteins can also bind consensus motifs and interfere with the start of transcription. This interference can be accomplished by preventing other transcription factor complexes from binding DNA and inducing transcription or by preventing the transcription start machinery from moving down the APOE promoter (Herdegen and Waetzig 2001).

We determined that inhibition of JNK induced APOE promoter activation in the proximal apoE promoter region -300/ +86. Recent examinations of the APOE promoter revealed a potential AP-1 binding site in the proximal promoter (Gafencu et al. 2007; Maloney et al. 2007). One study employed DNA-binding experiments to show that c-Jun binds the APOE promoter downstream of position -55 (Gafencu et al. 2007). A second study examined an even smaller fragment of the APOE promoter (-15/ +45) and predicted that c-Jun binds the APOE promoter in this region (Maloney et al. 2007). We suggest that a potential AP-1 binding site on the APOE promoter region closest to the transcription start site interferes with transcriptional start machinery. The predicted AP-1 binding site is present at positions -8 to -1 (TTGAGTCC), downstream of the TATAA sequence, where RNA polymerase II starts to assemble the
transcriptional machinery. Thus, by inhibiting c-Jun activation and AP-1 binding, JNK inhibitors prevent interference of transcriptional start and promote APOE expression.

While several studies identified binding sites for c-Jun in the proximal APOE promoter, one study showed that overexpression of a dominant negative c-Jun by peripheral adenovirus-mediated gene transfer increased APOE mRNA levels in the liver and increased apoE plasma levels (Drosatos et al. 2007). This work further supports our findings that inhibition of c-Jun activation increases APOE expression.

We have previously shown that apoE protects against inflammatory signaling (Pocivavsek et al. 2009). ApoE has been implicated in modulating the CNS inflammatory response in an isoform-dependent manner (apoE2 > apoE3 > apoE4). ApoE4 carriers have an increased risk of developing AD, although the mechanisms for that increased risk have not been elucidated. Further, it has not been determined why apoE4 has poor anti-inflammatory properties compared to apoE2 or apoE3. ApoE4 has major structural characteristics that distinguish it from apoE2 or apoE3 due to the presence of arginines at amino acid positions 112 and 158. The Arg112 affects the conformation of Arg61 side chain and a domain interaction between the carboxyl-terminal and amino-terminal domains of apoE4 (Dong and Weisgraber 1996). The changes in apoE4 structure diminish the stability of apoE4 (Hatters et al. 2006) leading to preferential degradation of apoE4 by glial cells (Riddell et al. 2008). In APOE ε2, APOE ε3, and APOE ε4 targeted replacement mice, levels of apoE protein vary with decreased level of apoE in APOE ε4 targeted replacement mice (Riddell et al. 2008; Vitek et al. 2007). These findings suggest that the apoE isoform associated risks may
be related to the expression of apoE protein. Increasing apoE protein maybe an effective way to enhance the anti-inflammatory properties of apoE.

Research has also focused on the influence of apoE on amyloid deposition. *In vitro* and *in vivo* studies have shown that apoE-containing lipoprotein particles can bind to Aβ, promoting its clearance and degradation (DeMattos et al. 2004; Koistinaho et al. 2004; Manelli et al. 2004). Further, LXR agonists, which increase apoE levels, have been found to decrease Aβ levels in APP transgenic mice (Burns et al. 2006; Koldamova et al. 2005b; Riddell et al. 2007). On the other hand, evidence suggests that apoE promotes deposition of Aβ in older mice (Fagan et al. 2002; Holtzman et al. 2000; Irizarry et al. 2000). Transgenic APP<sup>V717F</sup>+/− mice expressing no apoE have significantly less fibrillar Aβ compared to APP<sup>V717F</sup> expressing mouse apoE or human apoE2, apoE3, or apoE4 (Fagan et al. 2002). Among the mice expressing human apoE, isoform-specific effects were observed; APOE ε4 mice had more Aβ deposits than APOE ε3, which had more Aβ deposits than APOE ε2 mice (Fagan et al. 2002; Holtzman et al. 2000). These studies propose that increasing apoE may promote Aβ fibrillation. Thus, this work demonstrating that apoE can be increased by JNK inhibitors, which would have positive anti-inflammatory effects, may also positively or negatively affect Aβ accumulation in the brain.

A number of pharmacological JNK inhibitors have been discovered that target various portions of the JNK signaling pathway (Manning and Davis 2003). Compounds that inhibit JNK have entered clinical trials to treat leukemia and other cancers (Bogoyevitch and Arthur 2008; Manning and Davis 2003). In the CNS,
various injuries trigger activation of JNKs, including pathological entities in AD (Marques et al. 2003; Morishima et al. 2001), Parkinson’s disease (Crocker et al. 2001; Saporito et al. 2000), and ischemic injury (Kuan et al. 2003). Targeting JNK inhibition may prove to protect against neurodegenerative insults, by either inhibiting JNK activation in neurons associated with increased apoptosis or preventing the inflammatory response in glial cells. We used two different compounds, L-JNK1 and SP600125, to inhibit JNK and both increased APOE mRNA and protein. We suggest that JNK inhibitors may prove useful by increasing apoE and its protective anti-inflammatory properties.
Figure 12: Activation of glia by LPS increased NO production in a JNK-dependent manner. A) In primary mixed glial cultures, LPS promoted nitrite accumulation in the conditioned media at 24 hr (Student’s T-Test, mean ± SEM; ***P < 0.001; n = 4). B) Primary glial cultures were treated with indicated doses of a JNK
inhibitor (SP600125), ERK inhibitor (PD98059), or PI3K/Akt inhibitor (Wortmannin) and stimulated with 100 ng/ml LPS for 24 hrs. SP600125 treatment reduced nitrite production in a dose-dependent manner. PD98059 and Wortmannin did not effect LPS-induced nitrite production (One-Way ANOVA using Newman-Keuls Multiple Comparison Test for posthoc analysis, mean ± SEM; ## P < 0.01, ### P < 0.001 compared to indicated cultures; n = 3). C) Primary glial cultures were treated with 10µM SP600125 or 1µM L-JNK1 and stimulated with LPS for 24 hrs. Both JNK inhibitors reduced nitrite production (One-Way ANOVA using Newman-Keuls Multiple Comparison Test for posthoc analysis, mean ± SEM; ***P < 0.001 compared to control culture; ###P < 0.001 compared to indicated cultures; n = 4).
Figure 13: Glial activation decreased apoE production and secretion. Primary mixed glial cultures were treated with 100 ng/ml LPS for 24 hrs. A) APOE mRNA was measured by Real-Time PCR. Relative Quantity of APOE expression shows that LPS treatment reduced APOE mRNA (Student’s T-Test, mean ± SEM; *** P < 0.001; n = 6). B) Cell lysates and conditioned media were analyzed by Western blotting with antibodies to apoE and β-actin. Representative blots show that LPS reduced apoE
levels in the cell lysate and conditioned media. C) Western blot data were quantified
as percent of control apoE in the cell lysate or media. Cells treated with LPS showed a
decrease in lysate apoE and secreted apoE compared with control untreated cells
(Student’s T-Test, mean ± SEM; * P < 0.05, *** P < 0.001 compared to corresponding
control cultures; n = 6).
Figure 14: Inhibition of JNK increased apoE production and secretion in glia.

Primary mixed glial cultures were treated with JNK inhibitors, SP600125 (10uM) or L-
JNK1 (1uM), for 24 hrs. A) APOE mRNA was measured by Real-Time PCR. Relative Quantity of APOE expression shows SP600125 increased APOE mRNA (Student’s T-Test, mean ± SEM; ** P < 0.01; n = 6). B) Cell lysates and conditioned media were analyzed by Western blotting with antibodies to apoE, ABCA1, and β-actin. Representative blots show that SP600126 (SP) and L-JNK1 (L-J) treatment increased apoE and ABCA1 levels in the cell lysate and increased apoE in the conditioned media. C) Western blot data were quantified as percent of control apoE in the cell lysate. Cells treated with SP600125 or L-JNK1 showed increased lysate apoE compared with control untreated cells (Student’s T-Test, mean ± SEM; * P < 0.05 compared to corresponding control cultures; n = 6 for SP600125 treatment and n = 3 for L-JNK1 treatment).
Figure 15: Stimulation of APOE promoter activity by inhibiting JNK.  
A) Schematic representations of APOE promoter constructs used in our studies.  
B) BV2 cells were transfected with various APOE promoter constructs and luciferase activity was measured.  
JNK inhibitor SP600125 (10uM) treatment increased luciferase activity
for all constructs compared to untreated control for each construct (Student’s T-Test, mean ± SEM; * P < 0.05, ** P < 0.01, *** P < 0.001 compared to corresponding controls; n = 3).
Figure 16: Inhibition of JNK in vivo increased apoE. 5µl or 10µl SP600125 (10mM) or vehicle control (5 or 10 µl) was injected into the hippocampus of adult
mice. After 24 hrs, the cortices and hippocampi were dissected and homogenized for Western blot analysis. A) Analysis by Western blot revealed that apoE levels in the ipsilateral hippocampus increased with SP600125 (SP) but remained unchanged in the cortex. β-actin levels remained unchanged. B) Western blot analysis showed that apoE and ABCA1 levels were similar in the contralateral hippocampus from animals injected with either vehicle (C), 5µl or 10µl SP600125 (SP). ApoE and ABCA1 levels in the ipsilateral hippocampus increased in animals injected with SP. Levels of β-actin remained unchanged. D) Quantification of Western blot analysis revealed a significant increase in apoE with SP600125 (One-Way ANOVA using Newman-Keuls Multiple Comparison Test for posthoc analysis, mean ± SEM; * P < 0.05, *** P < 0.001 compared to vehicle control injected animals; n = 4 for vehicle control, n = 4 for 5µl SP600125, n = 2 for 10µl SP600125 injection).
Figure 17: Model of APOE gene regulation by JNK, c-Jun, and AP-1. We suggest that LPS treatment increases phospho-JNK, resulting in activation of downstream c-Jun and promoting AP-1 to bind the APOE promoter. AP-1 binding the APOE promoter downstream of the TATAA sequence interferes with transcriptional start and inhibits transcription of APOE mRNA.
Chapter V: CONCLUSIONS AND DISCUSSION

A. CONCLUSIONS

1) LDL receptor family has immunomodulatory signaling properties in microglia
   • EP-induced activation of LDL receptors decreases JNK activation
   • Inhibition of JNK overcomes the LPS-induced increase in nitric oxide accumulation

2) Inflammatory response in microglia reduces apoE expression
   • Inhibition of JNK overcomes the LPS-induced decrease in apoE production

3) LRP1 regulates microglial inflammation
   • Inhibition of JNK is essential to overcome the LPS-induced increase in nitric oxide in both wild-type and LRP -/- microglia
   • LRP1 activation by EP treatment reduces phosphorylation of JNK

4) ApoE expression is regulated by JNK
   • Activation of JNK decreases APOE mRNA and apoE protein
   • Inhibition of JNK increases APOE promoter activity, APOE mRNA, and apoE protein
   • JNK inhibition increases apoE in vivo
B. DISCUSSION

ApoE Signaling Properties \textit{In Vivo}

In this thesis research, we have reported that treatment of microglia with EP decreased JNK activation and increased ERK activation. We were particularly interested in the effects on JNK activation because the effects of EP on JNK inhibition were mediated via LDL receptor family members, specifically LPR1, since in microglia lacking LRP1, the effects of EP on JNK signaling were attenuated. These are the first studies to identify signaling properties of LDL receptor family members in microglia.

This work in microglia complements our earlier work examining the effects of EP on neurons. We have previously studied the effects of EP on ERK activation and JNK inhibition in neurons \textit{in vivo} (Hoe et al. 2006). By injecting EP or apoE into the rat hippocampus we modeled transient increases in apoE that maybe observed after neuronal damage (Poirier et al. 1991). For these studies, we injected 2µM EP into the rat hippocampus and examined the ipsilateral hippocampal lysates for activation of ERK and JNK at up to 7 days post-injection. We showed that in a time-dependent manner, EP increased ERK activation and reduced JNK activation in neurons. These findings \textit{in vivo} complement the effects of EP in cultured neurons (Hoe et al. 2005) However, we did not analyze changes in kinase activation in glial cells. Thus, we set out to examine the effects of EP on JNK and ERK phosphorylation in microglia, Our most recent work shows that EP reduced phosphorylation of JNK and increased phosphorylation of ERK in microglia, perhaps giving us new insight into our \textit{in vivo}
studies. We now conclude that the signaling effects of EP that we saw in vivo may also be attributed to changes in microglial signaling.

**Application of EP Reduces Endogenous ApoE**

Because of their anti-inflammatory properties, apoE mimetic peptides have been studied as therapeutic approaches for animal models of head injury (Laskowitz et al. 2007; Lynch et al. 2005), multiple sclerosis (Li et al. 2006), and intracerebral hemorrhage (James et al. 2009). Our work supports these findings and, in addition, we have identified some of the signaling cascade mechanisms that mediate the anti-inflammatory effects of the apoE mimic, EP. However, we also show that application of EP reduced endogenous apoE expression in a dose-dependent manner in vitro and in vivo (Fig. 18). In BV2 microglia, application of increasing doses of EP reduced apoE expression in the cell lysates. A significant reduction in apoE was observed with application of 10µM and 20µM EP (Fig. 18A), which are more than 10-fold higher than endogenous apoE present in the brain. We also injected EP into the hippocampus of 3xTg mice, a mouse model that overexpresses three genetic mutations implicated in AD (APP, tau and presinilin-1). In this model, we found that injection of EP reduced endogenous apoE expression in a dose-dependent manner (Fig. 18B). Quantification of the Western blot analysis showed that injection of 10µM EP significantly reduced apoE by 65% in vivo (Fig. 18C).

Pharmacologic inhibition of JNK increased apoE production in cultured glial cells and *in vivo* (Chapter IV). Taken together, these data suggest that activation of apoE receptors should increase apoE expression. Thus, we were surprised to find that EP reduced endogenous apoE expression in a dose-dependent manner in BV2 cells and in the mouse brain (Fig. 18). We speculate EP could affect other signaling pathways (perhaps even beyond those linked to the LDL receptor family) and some of these effects could run counter to the effects of EP on JNK inhibition and apoE expression.

At high doses of EP, there could be additional signals to cellular machinery that regulates cholesterol homeostasis that there is ample apoE present and apoE transcriptional machinery is inhibited. As apoE-mimetic compounds are studied as therapeutic approaches to various CNS injuries, it is important to consider the changes that apoE-mimetic peptide treatment may have on endogenous apoE levels.

**Regulation of ApoE Expression by Cellular Cholesterol Homeostasis Machinery**

Cholesterol homoeostasis is transcriptionally regulated by sterol regulatory element-binding proteins (SREBPs) and LXRs. SREBPs are transcription factors that regulate synthesis and endocytosis of cholesterol and fatty acids. LXRs are nuclear receptors that regulate transcription of various genes involved in lipid efflux and transport from cells (Zelcer and Tontonoz 2006). ABCA1, a cholesterol transport pump, and APOE are two genes that are regulated by LXR. These nuclear hormone receptors are activated by oxysterols, including 24(S)-hydroxycholesterol and 22(R)-hydroxycholesterol. Ligand binding causes LXRs to form heterodimers with 9-cis-
retinoic acid receptor (RXR), which together regulate gene expression. While LXR is expressed in neurons and glia throughout the brain (Wang et al. 2002; Whitney et al. 2002), activation of LXR by pharmacological agonists predominantly affects gene expression and cholesterol efflux in glial cells (Whitney et al. 2002). With implications to AD, several studies have focused on LXR agonists as pharmacological targets to promote Aβ clearance (Burns et al. 2006; Jiang et al. 2008; Koldamova et al. 2005a; Koldamova et al. 2005b; Riddell et al. 2007; Sun et al. 2003). Reductions in Aβ coincided with induction of ABCA1 and apoE expression by LXR agonists (Jiang et al. 2008; Riddell et al. 2007). In our studies, we found that JNK inhibition increased both apoE and ABCA1. We suggest that by inhibiting c-Jun activation and AP-1 binding to the proximal APOE promoter, JNK inhibitors prevent a basal level of interference at the transcriptional start site, and thus promote APOE expression. It would be interesting to test whether a similar mechanism could explain the upregulation of ABCA1, examining whether JNK inhibition regulates ABCA1 expression directly by targeting the ABCA1 promoter. ABCA1 and apoE are co-regulated under several conditions in glia, which is consistent with the fact that their functions in lipid efflux are dependend on each other (Hirsch-Reinshagen et al. 2004).

**Therapeutic Targeting of JNK Inhibition in the Brain**

Our work implicates JNK inhibition as a target to increase apoE protein levels and inhibit inflammation in the brain. We have completed two independent studies that induced JNK inhibition *in vivo*: injections of EP or apoE into the rat hippocampus...
resulted in decreased phospho-JNK (Hoe et al. 2006) and injections of JNK inhibitor into the mouse hippocampus increased apoE (Chapter IV).

In the brain, three isoforms of JNK are expressed. While JNK1 and JNK2 are extensively expressed throughout tissues, the JNK3 isoform is selectively expressed in the brain, heart, and testis (Martin et al. 1996; Yang et al. 1997). The high expression of JNK1 and JNK2 systemically makes these isoforms poor targets for pharmacological inhibition because losing their functions may be detrimental to peripheral targets (Resnick and Fennell 2004). Development of specific JNK3 inhibitors is preferable for the treatment of CNS disorders. The specific roles of JNK3 in the CNS have been partially addressed using JNK3-deficient mice (Yang et al. 1997). JNK3 mediates stress-induced cell apoptosis (Kuan et al. 2003; Yang et al. 1997). Mice lacking JNK3 were resistant to glutamate receptor agonist kainic acid, showing reduced seizure activity and neuronal apoptosis in the hippocampus after excitotoxic damage (Yang et al. 1997). JNK3-deficient mice were also protected from brain injury after a model of cerebral ischemia (Kuan et al. 2003). In these studies, phosphorylation of c-Jun and activation of AP-1 were significantly reduced in the JNK3-deficient animals, suggesting that the neuroprotection observed in this animal model was due to elimination of a JNK3-mediated signaling cascade (Kuan et al. 2003; Yang et al. 1997).

While these studies have focused on the role of JNK3 in neuronal injury, JNK3 is present in glial cells as well (Hidding et al. 2002). We propose that targeted inhibition of JNK3 may also protect against glial inflammation. Our studies show that
two JNK inhibitors, L-JNK1 and SP600125, both overcome LPS-induced accumulation of NO in microglia (Pocivavsek et al. 2009) or mixed glial cultures (Chapter IV). These inhibitors target all three JNK isoforms. In the future, we would like to determine whether JNK-mediated glial activation can be overcome by inhibition of JNK3 alone. Current drug discovery initiatives are working to develop a selective JNK3 inhibitor, a particularly challenging task given that these small molecule inhibitors function by competitively interacting at the ATP-binding site that is relatively similar across isoforms (Resnick and Fennell 2004). Discovering compounds that selectively prevent the actions of JNK3 will be invaluable to further understanding the role of JNK3 in neurons and will also help elucidate the role of JNK3 in glial cells.

We have also found that LPS-induced activation of JNK decreased apoE in microglia and mixed glia, but we do not know which JNK isoform mediates these effects. In our studies, LPS treatment decreased APOE mRNA and protein. Inhibition of JNK overcame LPS-induced changes to apoE and increased basal APOE mRNA and protein. Glia from JNK3-deficient mice could be used to determine whether LPS-induced activation of these cultures differs from control LPS-stimulated cultures. If JNK3 is primarily responsible for mediating effects on inflammation, we predict that JNK3-deficient cells will be protected from LPS-induced activation of NO production, JNK3-deficient glial cells may also be protected from the LPS-induced decrease in apoE. These would be very interesting studies and further our understanding of the precise mechanism by which JNK inhibition increases apoE.
Contrasting Functions of LRP1 Ligands tPA and EP

Our work is the first to show LRP1-mediated signaling in microglial cells. EP treatment of LRP1-expressing microglia decreased JNK activation and significantly attenuated LPS-induced JNK activation. While LRP1-deficient microglia showed similar JNK activation when stimulated with LPS, EP treatment did not attenuate the effects of LPS in LRP1-deficient microglia. Our study is the second to employ LRP1-deficient microglia. The other study focused on an interaction between tPA and LRP1 in microglia (Zhang et al. 2009). Their work suggests that an interaction between tPA and LRP1 mediates the deleterious effects of tPA. In their model, middle cerebral artery occlusion (MCAO), a model of cerebral ischemia, induced microglial activation. tPA-deficient animals, as well as animals that lack LRP1 in microglia, were protected from MCAO-induced microglial activation. Exogenous application of tPA to tPA-deficient animals increased microglia activation, but animals that lacked LRP1 in microglia did not show tPA-induced microglia activation with exogenous tPA application (Zhang et al. 2009). To address a possible mechanism for the pro-inflammatory effects of LRP1, the authors studied changes in microglial activation by measuring accumulation of iNOS and nitrotyrosine. Using primary microglial cultures, they showed that LRP1-deficient microglia exposed to glucose-oxygen deprivation, an in vitro model of ischemic injury, had significantly less iNOS expression when compared to wild-type cultures. In vivo immunohistochemical and Western blot analyses revealed that nitrotyrosine formation was decreased in microglial LRP1-deficient animals compared to wild-type animals after MCAO. The
authors speculate that tPA induces LRP1-mediated microglial activation by inducing iNOS, synthesis of nitric oxide, and accumulation of nitrotyrosine. Our work challenges their report that LRP1-deficient microglia do not accumulate iNOS and produce NO after activation. While they worked with an ischemic injury model, we report that LRP-deficient microglia and wild-type microglia respond similarly to LPS stimulation with nitrite accumulation and iNOS. While these studies differ in their methods for microglial activation and LRP1 activation, our work suggests that LRP1 activation downregulates the microglial inflammatory response and that LRP1 activation is a mechanism to inhibit inflammation.

**Proteolytic Processing of LRP1 Promotes Its Anti-Inflammatory Signaling**

We have reported that the proteolytic cleavage of the LDL receptor family members in neurons promotes receptor-mediated signaling (Hoe et al. 2005) and we now propose a similar mechanism in microglia. The cytoplasmic tail of LRP1 interacts with adaptor protein JIP (Gotthardt et al. 2000) and JIPs have been implicated as inhibitors and activators of JNK signaling (Dickens et al. 1997; Mooney and Whitmarsh 2004; Whitmarsh et al. 2001; Willoughby et al. 2003). We speculate that with apoE-induced LRP1 activation in microglia, LRP1 is cleaved to release the cytoplasmic tail of LRP1 and JIP from the cell membrane. If JNK is localized elsewhere subcellularly, then this release would allow JIP to modulate activation of JNK. LRP1 is proteolytic processed by gamma-secretase to generate an intracellular LRP1 fragment (May et al. 2002) and soluble forms of extracellular LRP are found in
the plasma and cerebral spinal fluid (Quinn et al. 1997; Quinn et al. 1999). Though we have not studied the effects of EP on proteolytic processing of LRP1, we speculate that similar to ApoEr2 (Hoe and Rebeck 2005), EP increases proteolytic processing of LRP1.

LRP1 proteolysis by gamma-secretase induces anti-inflammatory signaling by releasing the intracellular domain (ICD) of LRP1 in peritoneal macrophages. LRP1-ICD translocates to the nucleus and inhibits the interferon-gamma promoter (Zurhove et al. 2008). Studies with LRP1-deficient macrophages showed that basal transcription of LPS target genes is increased compared to wild-type cells (Zurhove et al. 2008). For our studies in microglia, we found that LPS stimulation similarly increased NO production in wild-type and LRP1-deficient cultures, but we did not examine other LPS target genes that might account for an increase in basal inflammatory response in microglial cells. Overall, however, this study supports our hypothesis that LRP1 modulates inflammatory signaling. We believe that apoE-mediated activation of LRP1 reduces JNK activation and we aim to determine in the future whether the reduction in JNK signal is dependent on gamma-secretase cleavage of LRP1. We can also study whether the interaction between tPA and LRP1 perhaps does not promote the same proteolytic cleavage of LRP1, which would prevent the release of LRP1-ICD to regulate expression of inflammatory proteins.

Our work showed that activation of LRP1 by an apoE mimetic reduces the microglial inflammatory response. For anti-inflammatory approaches, it may be more important to focus on LRP1 rather than on enhancing LRP1 proteolysis by gamma-
secretase. Gamma-secretase is a multi-subunit protein complex that cleaves proteins within the transmembrane domain. APP and Notch are two of the most well studied substrates of gamma-secretase cleavage, but currently over 20 targets have been identified (Sisodia and St George-Hyslop 2002). Increased processing of APP by beta-secretase and gamma-secretase results in accumulation of toxic Aβ. Increased Notch proteolysis by gamma-secretase has been implicated in many cancers (Gordon et al. 2008). Thus, gamma-secretase inhibitors are in clinical trials as treatments of AD and various cancers (Wolfe 2008) and it would be potentially very harmful to try to increased gamma-secretase under such conditions. Considering the anti-inflammatory signaling induced by gamma-secretase processing of LRP1 (Zurhove et al. 2008), inhibition of gamma-secretase may detrimentally affect functions of LRP1. Drug discovery initiates should aim to design gamma-secretase inhibitors that target a specific substrate implicated in a disease, limiting detrimental side effects resulting from ubiquitous inhibition of gamma-secretase activity.

**Overall Conclusions**

Our studies demonstrate that apoE-induced anti-inflammatory properties involve the JNK signaling pathway. We show that the anti-inflammatory properties of apoE can be promoted through activation of LDL receptor family member LRP1. This work is consistent with reports that apoE has anti-inflammatory properties in the CNS (Laskowitz et al. 1997; Laskowitz et al. 2000; Laskowitz et al. 1998; Laskowitz et al. 2007; Laskowitz et al. 2001; Lynch et al. 2001; Lynch et al. 2003; Lynch et al. 2005;
Mace et al. 2007). Our data show that an apoE mimetic peptide, EP, reduced phosphorylation of JNK in microglia. We also find that by modulating JNK activation, EP attenuated the LPS-induced inflammatory responses of increased NO production and decreased apoE. We pursued inhibition of JNK as an approach to increase endogenous apoE, which might further the anti-inflammatory properties of JNK inhibition. Our data show that JNK inhibition increased APOE promoter activity, APOE transcription, and apoE protein. Taken together, these data support a model of apoE immunomodulatory signaling (see Fig. 19), suggesting that through a negative feedback loop, apoE reduces microglial JNK activation and this reduction regulates the expression of apoE. While this feedback loop regulates glial inflammation by reducing JNK activation and increasing apoE expression, *in vivo* other factors may interfere and disrupt this regulation.
**Figure 18:** ApoE peptide reduced apoE levels in a dose-dependent manner *in vitro* and *in vivo*. A) BV-2 microglia were treated with 0.1uM, 1uM, 10µM or 20µM EP for 24 hours. Quantification of Western blot analysis shows that EP treatment reduced apoE expression in a dose dependent manner. B) 3xTg mice were injected with PBS control, 1µM EP, or 10µM EP and sacrificed 24 hrs later. Homogenized brain lysates were analyzed by Western blot for apoE expression. Animals treated with 1µM EP and 10µM EP had less apoE expression than control treated animals. C) Quantification of Western blot analysis shows that *in vivo* injection of EP decreased apoE expression in a dose dependent manner.
Figure 19: A schematic model of apoE immunomodulatory signaling. ApoE-induced activation of LRP1 decreases phospho-JNK. A reduction in phospho-JNK regulates APOE promoter activity by increasing APOE transcription. Increased apoE can modulate microglial cell activation by regulating LRP1 activation.
Supplemental Figure 1: Immunostaining of primary microglia with microglial markers OX42 and Iba1. A. Primary microglia were stained with rabbit polyclonal OX42 / Texas Red or OX42 / Alexa 488. Microglial cell purity was confirmed to be > 95%. B. Primary microglia were stained with mouse monoclonal Iba1 / Alexa 488.
Supplemental Figure 2: Expression of functional LDL receptor family members in BV2 microglia and primary mixed glia. A) BV2 microglia and primary mixed glia were treated with FITC-labeled α2M* and ligand endocytosis was detected.
intracellularly by microscopy (left panels). Cells were pretreated with 50 nM RAP for 10 min and then treated with FITC-labeled α2M* (right panels) and ligand endocytosis was drastically decreased. B) Expression of the LDL receptor family members, ApoEr2, VLDLR, LDLR, and LRP was detected by Western blot analysis comparing primary neuronal cultures (N) and primary mixed glial cultures (G). Glial cells express VLDLR, LDLR, and LRP.
LIST OF PUBLICATIONS

Pocivavsek, A, Rebeck, GW. Inhibition of c-Jun N-terminal kinase increases apoE in vitro in primary glia and in vivo in mouse brain. *In preparation.*

Pocivavsek, A, Mikhailenko, I, Strickland, DK, Rebeck, GW. Microglial low-density lipoprotein receptor-related protein 1 modulated c-Jun N-terminal kinase activation. *In preparation.*


Christie RH, Chung H, Rebeck GW, Strickland D, Hyman BT. 1996. Expression of the very low-density lipoprotein receptor (VLDL-r), an apolipoprotein-E receptor,


neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. Cell 97(6):689-701.


