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Neuroinflammation in Alzheimer’s Disease: Characterization and Modification of the Response of Transgenic Mice to Intrahippocampal Lipopolysaccharide Administration

Donna Lorraine Herber

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Neuroinflammation in Alzheimer’s Disease:

Characterization and Modification of the Response of Transgenic Mice to Intrahippocampal Lipopolysaccharide Administration

by

Donna Lorraine Herber

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Keywords: microglia, astrocyte, nicotinic receptor, amyloid, dexamethasone

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Dedication

This work is dedicated to the memory of Leo William Herber, Jr. We miss you Dad.
Acknowledgements

All of the studies described herein were a collaborative effort. I greatly appreciate the training I received upon entering the program from Mary Pacheco, Javier Cuevas, Giovanni DiCarlo, Donna Wilock, Chad Dickey, and Emily Severance. Excellent technical support was also provided by Jessica Maloney, Lisa Roth, Menchu Barcenas, Keisha Symmonds, and Jennifer Alamed. The mouse colony was maintained by Nedda and David Wilson, Jeri Mason, and Melissa Freeman. I would like to say a special thank you to Marcia Gordon and Dave Morgan for providing me with resources, support, and independence to conduct my research. This work was supported by NIH grants AG15490 and AG18478.
# Table of Contents

List of Tables iii

List of Figures iv

Abstract vi

Introduction 1

Chapter One: Biochemical and Histochemical Evidence of Nonspecific Binding of $\alpha 7$ nAChR Antibodies to Mouse Brain Tissue 10
   Abstract 10
   Introduction 11
   Materials and Methods 13
   Results 18
   Discussion 31

Chapter Two: Acute and Chronic Microglial Inflammatory Responses After Intrahippocampal Administration of Lipopolysaccharide 35
   Abstract 35
   Introduction 36
   Materials and Methods 38
   Results 45
   Discussion 60

Chapter Three: Time-Dependent Reduction in $\alpha$β Levels After Intracranial LPS Administration in APP Transgenic Mice 65
   Abstract 65
   Introduction 66
   Materials and Methods 68
   Results 71
   Discussion 82
Chapter Four: Dexamethasone Suppresses LPS-Induced Microglial Activation and Amyloid Clearance in APP Transgenic Mice

Abstract 85
Introduction 86
Materials and Methods 87
Results 93
Discussion 105

Conclusions 108

References 144

About the Author End Page
List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Primary Antibodies Used for Immunodetection (Ch. 1)</td>
<td>18</td>
</tr>
<tr>
<td>Table 2</td>
<td>Reverse Transcription Master Mix and Cycling Conditions (Ch. 2)</td>
<td>41</td>
</tr>
<tr>
<td>Table 3</td>
<td>Primer Sequences for RNA Analysis (Ch. 2)</td>
<td>42</td>
</tr>
<tr>
<td>Table 4</td>
<td>Primary Antibodies Used for Immunohistochemistry (Ch. 2)</td>
<td>45</td>
</tr>
<tr>
<td>Table 5</td>
<td>Primary Antibodies Used for Immunohistochemistry (Ch. 3)</td>
<td>70</td>
</tr>
<tr>
<td>Table 6</td>
<td>Primary Antibodies Used for Immunohistochemistry (Ch. 4)</td>
<td>90</td>
</tr>
<tr>
<td>Table 7</td>
<td>Primer Sequences for RNA Analysis (Ch. 4)</td>
<td>92</td>
</tr>
</tbody>
</table>
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.</td>
<td>Immunolabeling by α7 antibodies in nontransgenic mouse brain</td>
<td>20</td>
</tr>
<tr>
<td>Figure 2.</td>
<td>Reactive astrocytes colocalize with Congophilic plaques in APP+PS1 mice</td>
<td>22</td>
</tr>
<tr>
<td>Figure 3.</td>
<td>Lipopolysaccharide stimulates astrogliosis and immunolabeling by anti-α7 antibodies</td>
<td>24</td>
</tr>
<tr>
<td>Figure 4.</td>
<td>Genotyping and RNA analysis of α7+ and α7- mice</td>
<td>26</td>
</tr>
<tr>
<td>Figure 5.</td>
<td>Immunohistochemical analysis of α7+ and α7- tissue</td>
<td>28</td>
</tr>
<tr>
<td>Figure 6.</td>
<td>Western blot analysis of α7+ and α7- tissue</td>
<td>30</td>
</tr>
<tr>
<td>Figure 7.</td>
<td>RNA analysis by qRT-PCR</td>
<td>47</td>
</tr>
<tr>
<td>Figure 8.</td>
<td>Time course of RNA expression</td>
<td>50</td>
</tr>
<tr>
<td>Figure 9.</td>
<td>Time course of microglial protein expression and morphology</td>
<td>53</td>
</tr>
<tr>
<td>Figure 10.</td>
<td>Quantitation of immunohistochemical results for microglial markers</td>
<td>55</td>
</tr>
<tr>
<td>Figure 11.</td>
<td>Time course of astrogliosis in response to LPS</td>
<td>57</td>
</tr>
<tr>
<td>Figure 12.</td>
<td>Time course of TLR4 levels after LPS injection</td>
<td>59</td>
</tr>
<tr>
<td>Figure 13.</td>
<td>Dose response of LPS stimulated Aβ removal</td>
<td>72</td>
</tr>
<tr>
<td>Figure 14.</td>
<td>Glial response to LPS</td>
<td>74</td>
</tr>
<tr>
<td>Figure 15.</td>
<td>Time-dependent removal of Aβ by LPS injection</td>
<td>76</td>
</tr>
<tr>
<td>Figure 16.</td>
<td>Time-dependent glial reaction to LPS injection</td>
<td>79</td>
</tr>
<tr>
<td>Figure 17.</td>
<td>Altered microglial morphology in response to LPS</td>
<td>81</td>
</tr>
<tr>
<td>Figure 18.</td>
<td>LPS injection reduced diffuse but not compact amyloid deposits and was reversed by dexamethasone</td>
<td>96</td>
</tr>
</tbody>
</table>
Figure 19. CD45 and CR3 are induced by LPS and inhibited by co-treatment with dexamethasone  

Figure 20. FcγRII/III and SRA are induced by LPS but not inhibited by co-treatment with dexamethasone  

Figure 21. LPS stimulated gene transcripts have a pattern similar to protein expression  

Figure 22. Autotoxic mechanisms in Alzheimer’s disease  

Figure 23. Microglial activation states  

Figure 24. Lipopolysaccharide signaling cascades  

Figure 25. Mechanism of LPS stimulated inflammation and Aβ removal  

Figure 26. Mechanism of LPS stimulated inflammation and Aβ removal, and inhibition by dexamethasone
Neuroinflammation in Alzheimer’s Disease:
Characterization and Modification of the Response of Transgenic Mice
to Intrahippocampal Lipopolysaccharide Administration

Donna Lorraine Herber

ABSTRACT

Alzheimer’s disease (AD) is pathologically characterized by amyloid plaques, neurofibrillary tangles, inflammation, and neurodegeneration. According to the amyloid hypothesis of AD, the central mediating event of the disease is the deposition of amyloid. The inflammation hypothesis of AD states that it is the inflammatory response to plaques and tangles, rather than the actual lesions, which causes the disease. Studies described here combine the two approaches into a single model. Four studies are presented using a basic protocol of intrahippocampal lipopolysaccharide (LPS) injection to stimulate inflammation in transgenic mice. The first study looked at alpha7 nicotinic receptors during the glial response to Abeta deposits and LPS. Reactive astrocytes which immunolabeled for alpha7 were co-localized with Congophilic deposits in APP and APP+PS1 mice, and increased after LPS injection. Unfortunately, LPS injection into alpha7 knock out mice revealed the alpha7 labeling to be nonspecific. The second study evaluated the time course of protein and gene expression after LPS injection into nontransgenic mice. This experiment identified both a transient and chronic microglial inflammatory response, with changes in cell morphology. The third study evaluated a similar time course in APP mice. Concurrent with the inflammatory response, transient
reductions in Abeta burden were seen, though compact plaque load was unaffected. The fourth and final study used dexamethasone to inhibit LPS-induced inflammation in APP mice. LPS injection reduced Abeta burden, but was completely blocked by dexamethasone co-treatment. Though dexamethasone inhibited LPS-induced CD45 and complement receptor 3 levels (markers of general microglial activation), dexamethasone had no effect on scavenger receptor A or Fc gamma receptor II/III levels. An overall hypothesis regarding LPS mediated reductions in Abeta can be proposed: It is not the presence of the LPS molecule, nor the upregulation of receptors involved in phagocytosis, but rather general glial cell activation that mediates Abeta removal. Thus, a phagocytic cell must not only bind Abeta (by various receptors) but must also be capable of engulfing the material (via general cell activation). Taken together, these studies suggest that some level of inflammation in AD is beneficial and responsible for maintaining a balance between amyloid deposition and removal.
Introduction

In the United States, there are two approved therapeutic approaches to Alzheimer’s disease (AD) therapy. The neurodegeneration seen in AD is mainly cholinergic, thus anticholinesterase drugs were developed to increase the half-life of acetylcholine in the synapse. These drugs temporarily overcome cholinergic dysfunction, but have no effect on neurodegeneration. Excitotoxicity has also been linked to the neurodegeneration of AD, thus n-methyl-d-aspartate (NMDA) receptor blockers have been developed. These two courses of therapy are only modestly effective long term, and other targets have been identified. At the center of new developments are various means of amyloid removal coupled with anti-inflammatory therapies. Amyloid-depositing transgenic mice are useful in developing working models of AD as well as targeting new therapies. Though amyloid deposition, inflammation, and cognitive impairment have been shown in various transgenics, there are limitations to the models, including a lack of neurofibrillary tangles, no apparent neuron loss, and minimal cholinergic deficits. It is possible that enhancing the inflammatory state in the brain of these mice would more closely resemble the pathology of AD. Lipopolysaccharide (LPS, endotoxin) stimulates the innate immune response, leading to gliosis in the central nervous system and has been used historically to induce inflammation. The goals of the current studies are to evaluate and modify the response of transgenic mice to LPS administration. Inflammation would
potentially alter the glial response, amyloid load, cholinergic function, and neuronal survival of these mice.

Alzheimer’s Disease

Described by Alois Alzheimer almost a century ago, AD is pathologically distinguished by amyloid plaques, neurofibrillary tangles, and neuron loss (Alzheimer, 1907). The roles of neurofibrillary tangles and amyloid deposits in neurodegeneration and dementia of AD are controversial.

Amyloid formation follows a series of steps beginning with the amyloid precursor protein (APP), a membrane associated protein (reviewed in Nunan & Small, 2002). Proteolytic cleavage of APP by secretases yields a variety of peptide products, some harmless, others neurotoxic. Activity by gamma and beta secretases yields a 40 or 42 amino acid product known as the amyloid β peptide (Aβ_{1-40} and Aβ_{1-42}) that is released extracellularly. The Aβ_{1-42} is the more insoluble form, which can form oligomers, fibrils, and ultimately diffuse and compact plaques. The biochemical pathways associated with Aβ neurotoxicity are diverse, including direct interaction with neuronal cell surface proteins, and indirect actions of free radical production and glial activation (Canevari et al., 2004; Small et al., 2001; Walsh et al., 2002). Compelling evidence supporting the key role of Aβ in the pathogenesis of AD are the genetic aberrations found in familial cases of the disease. Mutations in APP and the presenilins (PS1 and PS2) have been identified in early onset AD, leading to the development of animal models. Mice transgenic for human mutations of APP and PS1 have accelerated amyloid deposition and measurable cognitive deficits (Gordon et al., 2001). In these mice, anti-Aβ antibody and nonsteroidal anti-inflammatory drug (NSAID) therapies led to reductions in Aβ and
behavioral improvements (Jantzen et al., 2001; Morgan et al., 2000; Wilcock et al., 2003, 2004a,b). Human trials using an active vaccination with Aβ<sub>1-42</sub> are also showing promising results for halting the cognitive decline process, providing further evidence for the amyloid hypothesis of AD (Hock et al., 2003; Schenk, 2004).

Neurofibrillary tangles are also abnormal deposits of insoluble fibrils which are formed in a similar aggregation cascade (Friedhoff et al., 2000). Tangle accumulation inside neurons begins with tau, a microtubule associated protein. Phosphorylation leads to dissociation of tau from the microtubules. Hyperphosphorylated tau can polymerize, ultimately forming characteristic tangles which prevail even after the host neuron dies. Tauopathy is not exclusive to AD. Frontotemporal dementias and Pick disease also exhibit tangles, as well as memory loss, personality changes, and extrapyramidal symptoms (Morishima-Kawashima & Ihara, 2002).

Neuronal loss in AD is grossly seen in the overall atrophy of the cortical and limbic systems of the human brain. Specifically, the basal forebrain cholinergic system is affected with overall loss of nicotinic and muscarinic receptors, as well as decreased acetylcholine release and choline acetyltransferase activity (Auld et al., 2002). The exact mechanism of cell death is probably a combination of factors. Accumulation of Aβ may lead to a localized autotoxic “innate immunoreaction” (McGeer & McGeer, 2002). Gliosis generates reactive oxygen species, cytokines, and chemokines, all potentially contributing to neurotoxicity (Akiyama et al., 2000). Direct action of Aβ on neurons has also been hypothesized to cause excitotoxicity, followed by neuronal death (Canevari et al., 2004). The preferential cholinergic loss in the disease may be attributable to the ability of Aβ to interact with alpha 7 nicotinic acetylcholine receptors (Bourin et al.,
Current approved therapies for human use target cholinergic deficits with anticholinesterase action (Frisoni, 2001). More recent therapies aim to prevent neuronal death via NMDA receptor blockade, preventing glutamate induced excitotoxicity (Reisberg et al., 2003).

The role of inflammation in AD is complicated. At the center of the controversy is whether inflammation results from the pathology of AD or contributes to it. Complex interactions between the complement cascade, cytokine and chemokine pathways, prostanoid mediated inflammation, and reactive oxygen species may all contribute to neurotoxicity (Akiyama et al., 2000).

Cell mediators of the inflammatory process include the neurons themselves, microglia, and astrocytes. Microglia, the resident macrophages of the brain (Kreutzberg, 1996), and astrocytes are activated in AD brain compared to non-demented controls, and are associated with amyloid deposits (Lue et al., 1996; Vehmas et al., 2003). In vitro experiments have demonstrated the ability of microglia to produce cytokines (interleukins IL1β and IL6, tumor necrosis factor TNFα, transforming growth factor TGFβ) and reactive oxygen species when stimulated with Aβ (Colton et al., 2000; Lue et al., 2001; Small et al., 2001). Immunohistochemical studies in human AD brain verify the presence of inflammatory cytokines associated with amyloid deposits (reviewed in McGeer & McGeer, 1995).

Supporting the role of inflammation in AD related cognitive impairment are epidemiological studies indicating decreased risk of AD associated with chronic nonsteroidal anti-inflammatory (NSAID) therapy (Gasparini et al., 2004; In ‘t Veld et al., 1998). Animal models concur, demonstrating decreased amyloid load after NSAID
treatment (Eriksen et al., 2003; Jantzen et al., 2002; Yan et al., 2003). Human trials have been less promising, possibly due to the short duration (6-12 months) of such trials (Gasparini et al., 2004; McGeer et al., 1996; Szekely et al., 2004).

**Alpha 7 Nicotinic Acetylcholine Receptors in AD Pathology**

Cholinergic dysfunction is a hallmark of AD neurodegeneration. Specifically, the basal forebrain cholinergic system is affected with overall loss of nicotinic receptors, as well as decreased acetylcholine release and choline acetyl transferase activity (Auld et al., 2002). The two main nicotinic acetylcholine receptors (nAChRs) in the human brain consist of α4β2 or α7 subunits, with smaller populations of α2-9 and β3-4 (Paterson & Nordberg, 2000). α7 nAChRs are ligand gated ion channels consisting of five identical subunits, which conduct both sodium and calcium ions. They are distinguishable from other nAChRs by affinity for α-bungarotoxin.

In human AD brain, nicotinic receptor populations have been extensively studied. α7 nAChR levels are generally decreased in AD, though there are variations in reports (Perry et al., 2001). Declines in receptors may be due to actual cell loss, or synaptic decline might account for the abnormalities. Age related changes in α7 nAChRs expression in APP transgenic mice have also been reported (Dineley et al., 2001, 2002a,b). Focus has been on α7 nAChRs due to the potential interaction of the receptor with Aβ peptides. Co-immunoprecipitation and receptor binding assays indicated Aβ1-42 binds α7 nAChRs with high affinity, but not α4β2 nAChRs, in both human and rat tissue (Wang et al., 2000a,b). Studies conducted with wild type α7 nAChRs indicate that Aβ1-42 blocks the receptor (Grassi et al., 2003; Lee et al., 2003; Liu et al., 2001; Pettit et al., 2001). Glial cells may also be involved in the interactions of Aβ1-42 and α7 nAChRs as
recent reports have shown expression of these receptors by reactive astrocytes in human AD brain in association with amyloid deposits (Teaktong et al., 2003).

Transgenic Mice

The pathological markers of AD are amyloid plaques, neurofibrillary tangles, inflammation, and neuron loss. Although genetic aberrations can account for only 5-15% of all AD cases, transgenic models have been developed that provide insight into the pathology. Four genes are associated with amyloid deposition (reviewed in Morishima-Kawashima & Ihara, 2002). Early onset familial AD has been associated with genes coding for APP, PS1, and PS2 (Goate et al., 1991; Levy-Lahad et al., 1995; Sherrington et al., 1995). Many APP mutations have been described, resulting in preferential cleavage by beta and gamma secretases, or by increasing formation of Aβ1-42, the more insoluble form of Aβ. The presenilins have been identified as components of the gamma secretase complex (De Strooper, 2003). In contrast, mutations in PS1 or PS2 lead to enhanced activity of gamma secretase, thus increasing production of Aβ. A susceptibility to AD has been associated with the apolipoprotein E4 allele, leading to increased amyloid deposition through an unknown mechanism (Corder et al., 1993). Mutations coding for tau have been identified in frontal temporal dementia and Parkinson’s disease, but not in AD. In humans these mutations result in the hyperphosphorylated state of tau, neurofibrillary tangles, and neuronal loss.

Transgenic mouse models of AD are limited in their pathology, but are useful for studying amyloid deposition. Mutated forms of human APP when expressed in transgenic mice leads to an age related deposition of amyloid and inflammatory gliosis. Mice transgenic for PS1 mutations do not show significant pathology. However, double
cross of the APP mouse line with PS1 yields accelerated amyloid deposition, inflammatory markers, down regulation of genes associated with learning and memory, and cognitive deficits, without neurotoxicity (Dickey et al., 2003; Duff et al., 1996; Gordon et al., 2001, 2002; Holcomb et al., 1998). The discrepancy between these transgenics and human AD brain lies obviously in the lack of neuron loss and tangles.

The specific role of the cholinergic system in AD may be explored using nicotinic receptor knockout mice. As previously discussed, the ability of Aβ to interact with α7 nAChRs makes this ion channel of particular interest. Mice transgenic for a homozygous null mutation of the α7 nAChR gene are viable and fertile, without any apparent gross or cellular nervous system disturbances, neurological or behavioral dysfunction (Orr-Urtreger et al., 1997; Paylor et al., 1998). A role for α7 nAChRs in learning and memory has been presumed, thus the lack of a behavioral phenotype in these mice may be due to compensation by other populations. In vivo examination of α7 nAChRs during inflammation- and Aβ- induced gliosis and neurotoxicity might be accomplished with these mice.

Lipopolysaccharide as a Neuroinflammatory Agent

Lipopolysaccharide (LPS) is a Gram-negative bacterial cell surface proteoglycan, also known as bacterial endotoxin, which triggers an inflammatory response by the host (Palsson-McDermott & O’Neil, 2004). LPS binds to circulating LPS binding protein, which subsequently transfers the LPS to soluble or cell membrane bound cluster differentiation marker CD14, a common receptor for bacterial components (Heumann & Roger, 2002). The CD14 protein does not contain an intracellular domain, so interaction with additional components is likely. Toll like receptor 4 (TLR4), as well as myeloid
differentiation protein MD2, form a complex through which LPS:CD14 can transduce a signal (Thomas et al., 2002). Multiple cascades are involved in the signal transduction, with nuclear factor NFκB as a key player (Sen & Baltimore, 1986). Downstream gene activation leads to the production of cytokines, reactive oxygen species, and prostanoids stimulating the cellular inflammatory response.

Historically, LPS has been used to mimic the inflammatory conditions seen in neurodegenerative diseases. Acute and chronic application of LPS into the brain ventricles of rats resulted in gliosis, cytokine production, cognitive deficits and neurotoxicity (Hauss-Wegrzyniak et al., 1998a,b, 2000, 2002; Willard et al., 1999). Pharmacological intervention with steroids, NSAIDs, antioxidants, or NMDA receptor antagonists attenuated some of the effects of LPS (Castano et al., 2002; Hauss-Wegrzyniak et al., 1999a,b; Jain et al., 2002; Kheir-Eldin et al., 2001; Szczepanik et al., 2003; Wenk et al., 2000, 2002; Willard et al., 2000).

With the introduction of amyloid depositing transgenics, LPS has been administered to accelerate Aβ deposition, activate glia, and trigger neurotoxicity (the latter a key component of AD that is lacking in these models). The experimental conditions of reports vary including the type and age of transgenic animals used; type, dose and route of administration of LPS; and post injection survival time. In some cases amyloid deposition was triggered (Qaio et al., 2001; Sheng et al., 2003; Sly et al., 2001), while in others amyloid clearance resulted (DiCarlo et al., 2001; Quinn et al., 2003). When considering the species of Aβ1-40 and Aβ1-42, the main indicator of Aβ reduction seems to be closely related to plaque load and survival time. Aged animals with significant amyloid deposits, surviving several days post injection, showed clearance of
Aβ deposits after LPS treatment. Compact plaques detected by either Congo red or thioflavin-S staining were inconclusively affected. *In vitro* studies show that microglia and as well as astrocytes react to LPS with phagocytic activity, as well as prostaglandin, cytokine, and nitric oxide production (Kalmar et al., 2001).

The ability of LPS to cause neuron death appears to be duration and region specific. Chronic infusion of LPS into the brain ventricles of rats resulted in neuron loss limited to the forebrain cholinergic nuclei (Hauss-Wegrzyniak et al., 1998a, 1998b, 2000, 2002; Willard et al., 1999). However, acute LPS injection into the brain parenchyma such as the anterior cortex and hippocampus did not triggered cell death (Kim et al., 2000; Herber et al., 2004a,b). An exception is the dopaminergic system which does show susceptibility to the degenerative effects of LPS (Kim et al., 2000; Castano et al., 2002).

Taken together, stimulating an inflammatory response in the brain of transgenic mice should lead to a better understanding of the pathogenesis of AD. Studies described herein address the effects of inflammation on gliosis and amyloid burden in these mice.
Chapter 1

Biochemical and Histochemical Evidence of Nonspecific Binding of α7 nAChR Antibodies to Mouse Brain Tissue

Abstract

Alpha 7 nicotinic acetylcholine receptors (α7 nAChRs) are involved in learning and memory, and are implicated in the pathology of Alzheimer’s disease and schizophrenia. Detection of α7 subunits can be accomplished via immunodetection or α-bungarotoxin binding techniques. In studies described here, standard protocols for immunohistochemistry and Western blotting were followed using several commercially available antibodies. Various mice were evaluated including nontransgenics, APP, PS1, APP+PS1, and α7 knockouts. Initial results with amyloid depositing mice revealed α7 immunolabeled astrocytes, in addition to expected neuronal staining. Subsequent studies with intrahippocampal injections of lipopolysaccharide into α7 knockout mice showed that both neuronal and astrocytic labeling by α7 antibodies was nonspecific. On Western blots of mouse brain proteins, none of the bands detected with antibodies directed against α7 subunits diminished in the α7 knockout mice. Although LPS related changes in the expression of some bands was found, these also were unaffected by the α7 genotype of the mice. In general, the Western staining patterns for these antibodies revealed few overlapping bands. These immunodetection data are in contrast to genotyping results and mRNA analyses which confirmed the disruption of the α7 allele, and lack of α7 message
in the knockouts. These findings suggest caution in interpreting results using several commercially available α7 nicotinic receptor antibodies.

Introduction

Cholinergic dysfunction is a hallmark of Alzheimer’s disease (AD) neurodegeneration. Specifically, the basal forebrain cholinergic system is affected with overall loss of nicotinic receptors, as well as decreased acetylcholine release and choline acetyltransferase activity (Auld et al., 2002). In vertebrates, the two main nicotinic acetylcholine receptors (nAChRs) in the brain are α4β2 and α7, with smaller populations of α2-10 and β3-4. α7 is a ligand-gated ion channel consisting of five identical subunits, with a high relative calcium permeability, and distinguishable from other central nervous system nAChRs by its affinity for α-bungarotoxin (McGehee & Role, 1995; Seguela et al., 1993).

In human AD brain, nicotinic receptor populations have been extensively studied. α7 levels are generally decreased in AD, though there are variations in reports (reviewed in Perry et al., 2001). Declines in receptors may be due to cell loss or synaptic decline. Age-related changes in α7 expression in mice transgenic for a human mutated Alzheimer precursor protein (APP) have also been reported (Dineley et al., 2001; 2002a,b). Particular focus has been on α7 due to the potential interaction of the receptor with Aβ peptides. Co-immunoprecipitation and receptor binding assays indicated Aβ1-42 binds α7 with high affinity, but not α4β2, in both human and rat tissue (Wang et al., 2000). Studies conducted with wild type α7 receptors indicate that Aβ1-42 blocks the receptor (Grassi et al., 2003; Lee et al., 2003; Liu et al., 2001; Pettit et al., 2001). Glial cells may also be involved in these interactions as recent reports have shown expression of α7 by
reactive astrocytes in human AD brain, particularly in association with amyloid deposits (Graham et al., 2002; Wevers et al., 1999; Teaktong et al., 2003).

In these experiments, we used standard protocols for immunostaining and Western blot analysis of α7 nAChR subunits in murine models of Alzheimer’s disease and neuroinflammation. Several commercially available antibodies were evaluated, and the results compared to genotyping and RNA analyses. Initially, four genotypes resulting from breeding transgenic mice carrying either mutant APP or presenilin 1 (PS1) transgenics were examined for α7 expression. Various reports in the literature led us to expect decreases in levels of nicotinic receptors in amyloid-depositing mice, such as the APP and APP+PS1 transgenics. Though no decreases in neuronal staining was seen, a surprising finding was α7-immunopositive astroglia in apposition with compact plaques in APP and APP+PS1 mice, but not PS1 or nontransgenics. In order to determine if amyloid was causing the astrocytic α7 expression, or if it was part of a more general inflammatory response, we injected LPS intrahippocampally into APP and nontransgenic mice. Immunohistochemical analysis revealed many α7 positive astrocytes in the injected animals, leading us to believe the expression was part of a general inflammatory response. In a final experiment using α7 null mice, the specificity of the antibodies was tested under both control and LPS stimulated conditions. No discernible differences were seen between α7+/+ versus α7-/- mice with any antibody used, regardless of procedure. Genotyping and RNA analyses confirmed the disruption of the α7 allele and lack of α7 message in the knockouts. We therefore conclude that commercially available antibodies against α7 as used in the methods detailed here fail to specifically detect the subunits.
Materials and Methods

Mouse Strains

Transgenic mice carrying an APP (Tg2576) and/or PS1 mutation were bred as described previously (Holcomb et al., 1998). Nontransgenic littermates were used as controls. Alpha-7-null mutant mice, originally described by Orr-Urtreger and colleagues in 1997, were derived from heterozygous breedings of animals purchased from Jackson Laboratories (Bar Harbor, ME). Nontransgenic and homozygous null mice from this cross were used in this study. Animals were group-housed under a 12 hr light-dark cycle with free access to chow and water. Experimental groups were balanced regarding age and gender.

Genotyping

APP and PS1 lines were analyzed as previously reported (Gordon et al., 2001). Mice from the α7 null mutation line were genotyped as follows: 2 mm diameter ear clips from the α7 heterozygous breedings were digested and the DNA extracted using Qiagen’s DNeasy® Kit (Valencia, CA), per the manufacturer’s instructions. Jackson Laboratories supplied the sequence of primers used to identify either the neo-cassette of the null mutation or the wild type allele, for use with the polymerase chain reaction (PCR):

Forward 5’CCTGGTCCTGCTGTGTTAAACTGCTTC3’; Reverse-WT (α7+)
5’CTGCTGGGAAATCCTAGGCACACTTGAG3’; Reverse-Neo (α7-)
5’GACAAGACCGGCTTCCATCC3’. Thermocycling conditions were as follows: 95°C for 4 minutes; 30 cycles of 95°C for 15 seconds, 60°C for 60 seconds, 72°C for
60 seconds; 72°C for 7.5 minutes; store at 4°C. PCR products were run on a 1% agarose gel, using ethidium bromide ultraviolet (UV) detection of bands at 440 bp (α7+) or 750 bp (α7-).

Intrahippocampal Injections

APP mice aged 16 months, α7 null mice aged 11 months, and their nontransgenic littermates were used in these studies. Mice were anesthetized using isoflurane and immobilized in a stereotaxic apparatus. One microliter injections of either saline or 4 µg/µl lipopolysaccharide (Salmonella abortus equi, Sigma, Saint Louis, MO) were delivered over a two minute period into the hippocampus (stereotaxic coordinates from bregma: -2.7 mm posterior; +/-2.5 mm lateral; -3.0 mm ventral). This procedure had been previously demonstrated in our lab to induce a neuroinflammatory response without adversely affecting animal survival (DiCarlo et al., 2001). All animal work was conducted under National Institute of Health guidelines, and approved by the University of South Florida’s institutional animal care and use committee. Animals were singly housed for the 7 day post treatment survival period under standard vivarium conditions.

Tissue Preparation

Mice were anesthetized with pentobarbital (200 mg/kg, ip), then perfused transcardially with 25 ml of saline. The right hemisphere of the brain was dissected into regions and stored at –80°C for subsequent biochemical analyses. For immunohistochemistry, left hemispheres were transferred into a 4% paraformaldehyde solution for 24 hr, then processed through a cryoprotection schedule of 10, 20, then 30% sucrose (24 hr in each solution). The tissue was sectioned horizontally on a sliding
microtome at 25 μm. Sections were then stored in Dulbecco’s phosphate buffered saline pH 7.4 (DPBS) with sodium azide (100 mM) at 4°C.

**RNA Analysis**

Mice obtained from the α7 heterozygous breedings were analyzed for mRNA using reverse transcription followed by PCR. The procedure was originally described in detail elsewhere (Dickey et al., 2003). RNA was extracted from the injected hippocampus of α7+ and α7- mice, using Qiagen’s Rneasy® procedure (Valencia, CA). RNA concentration was determined with Molecular Probes RiboGreen® RNA quantitation kit (Molecular Probes, Eugene, OR). Reverse transcription with mMLV (Invitrogen, Carlsbad, CA) was performed, and the resulting cDNA subjected to PCR using Amplitaq Gold (Applied Biosystems, Foster City, CA). Primers used to identify α7 were directed towards portions of exons 9-10: F5’GTGGGCTCTCAGTGGTCGT3’; R5’GTCCCCATCAGAGGGGTGTG3’. Thermocycling conditions were as follows: 95°C for 3 minutes; 45 cycles of 95°C for 15 seconds, 60°C for 60 seconds, 72°C for 60 seconds; 72°C for 7.5 minutes; store at 4°C. PCR products were run on a 1% agarose gel, using ethidium bromide UV detection of bands at 381 bp.

**Histology**

Immunohistochemical analysis of α7 nAChRs was performed using 25 μm free floating sections spaced 300 μm apart through the hippocampus. Details of this procedure were originally described elsewhere (Gordon et al., 1997). All steps were performed on a rotating platform at approximately 40 rpm, room temperature, unless otherwise stated. Sections were blocked for endogenous peroxidases (10% methanol,
3% hydrogen peroxide in DPBS) for 15 minutes, then washed 3 x 5 minutes with DPBS. The tissue was then permeabilized in a solution of 100 mM lysine, 0.2% triton x-100, and 4% normal goat or horse serum (Pel Freeze, Rogers, AK) in DPBS for 30 minutes. Sections were then incubated overnight in the appropriate primary antibody in DPBS and 4% serum, without shaking (Table 1). The following day, sections were incubated with shaking in primary antibody for one hour. Sections were then washed 3 x 5 minutes with DPBS, and then incubated in appropriate biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) at a concentration of 0.5 µg/ml in DPBS and 4% serum, for two hours. Sections were washed 3 x 5 minutes with DPBS, and then incubated for one hour in Vectastain® Elite® ABC solution (Vector Laboratories, Burlingame, CA), using 8 drops each of components A and B per 100 ml of DPBS. Sections were then washed 2 x 5 minutes with DPBS, followed by a single wash in tris buffered saline, pH 7.6 (TBS) for 5 minutes. The tissue was then incubated with a solution of 0.5% nickelous ammonium sulfate hexahydrate and 0.05% diaminobenzidine in TBS for 5 minutes. Color development was achieved by the addition of 0.03% hydrogen peroxide, and incubation for an additional 5 minutes, followed by three final washes. Controls for nonspecific binding of the secondary antibody were performed by excluding primary antibodies. Stained sections were mounted onto slides and air dried overnight. Slides were then processed through a dehydration schedule of 10 dips in water, followed by 2 x 3 minutes in each of 25%, 50%, and 75% ethanol, then 3 x 5 minutes in each of 95% ethanol, 100% ethanol, and Histo-Clear (National Diagnostics, Atlanta, GA). Slides were cover slipped with DPX (E.M. Sciences, Fort Washington, PA) and allowed to dry overnight.
In some cases, tissue stained with \( \alpha \)7 antibodies was counterstained with Congo red to verify location of compact plaque deposits. For this counterstain, immunostained sections were slide mounted and allowed to dry overnight. The slides were then hydrated for 30 seconds in water. Slides were then submerged for 20 minutes in a solution of 80% ethanol supersaturated with sodium chloride, then made alkaline with a final concentration of 0.01% sodium hydroxide. Slides were then incubated for 30 minutes in a separate portion of alkaline alcoholic saturated sodium chloride containing 0.2% Congo red dye (solution filtered prior to use). Slides were then dehydrated with 8 dips in 95% ethanol, then 8 dips in two baths of 100% alcohol. The tissue was finally run through 3 x 5 minutes of xylene and cover slipped with DPX.

**Western Blotting**

Mice obtained from the \( \alpha \)7 heterozygous breedings were analyzed for protein using SDS-PAGE followed by Western blot. Cerebral cortex previously stored at –80°C was homogenized in 10 mM HEPES buffer pH 7.4 containing a protease inhibitor cocktail (Roche, Indianapolis, IN). Crude protein concentrations were determined by the Bradford method using Bio-Rad Protein Assay Dye Reagent (Hercules, CA). Samples were denatured with Bio-Rad Laemmli sample buffer by boiling for 5 minutes. 10 \( \mu \)g of protein was loaded per well, and proteins separated using 10% polyacrylamide Bio-Rad Ready Gels. Bio-Rad Precision-Plus Protein™ All Blue molecular weight standards were run for band identification. The separated proteins were transferred to Immobilon™-P polyvinylidene fluoride membranes and immunoblotted (Millipore, Bedford, MA). Membranes were first blocked with 5% nonfat dry milk in borate-buffered saline pH 8.5 and 0.05% Tween-20 (BST) for one hour on a rocking platform at room temperature.
Membranes were then incubated with primary antibody in 2.5% nonfat dry milk in BST for one hour (Table 1). After washing 3 x 5 minutes with BST, blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:10000 dilution in 2.5% milk-BST for 30 minutes. Finally, membranes were triple washed in BST. Bands were identified using Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA) for chemiluminescent detection and subsequent film exposure for 0.5-5 minutes. The presence of α7 protein was verified by comparing the protein bands to the molecular weight standard markers. The expected molecular weight of the α7 subunit was 56 kDa.

Table 1. Primary Antibodies Used for Immunodetection

<table>
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<th>Immunohistochemistry Titer</th>
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<td>319</td>
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<td>H-302</td>
<td>Rabbit polyclonal</td>
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Results

Standard protocols for immunohistochemical and Western blot detection of α7 nAChR subunits were followed using several commercially available antibodies. These results were ultimately compared to genotyping and mRNA analyses of the α7 subunit. Initially, four genotypes resulting from breeding of APP and PS1 transgenics were
examined for α7 expression. Previous data had been published demonstrating the amyloid burden, gliosis, and behavioral phenotype of these mice (Gordon et al., 2001, 2002). The antibodies used for the current study included a rat monoclonal antibody (mAb 319), a mouse monoclonal (mAb 306) and a rabbit polyclonal (pAb H-302). In nontransgenic mice (Fig. 1), distinct ubiquitous positive immunostaining was seen throughout the brain with mAb 319. Regions analyzed included the dentate gyrus of the hippocampus, frontal cortex, striatum, and cerebellum. These results are consistent with previous reports (Dominguez del Toro et al., 1994).

*Figure 1. Immunolabeling by α7 antibodies in nontransgenic mouse brain.* Horizontal sections were immunostained using mAb 319 at a 1:5000 dilution. Ubiquitous neuronal stain was seen in all regions examined including the dentate gyrus of the hippocampus (A), cerebral cortex (B), striatum (C), and cerebellum (D). Scale bar = 100 µm.
Figure 1. Immunolabeling by α7 antibodies in nontransgenic mouse brain.
Mice transgenic for APP+PS1 were next analyzed. Distinct neuronal staining was noted with mAb 319 (not shown), similar to that seen in nontransgenics. A comparison of nontransgenic versus APP+PS1 mice revealed no differences in the intensity of neuronal staining in any region. In amyloid bearing regions such as the hippocampus and cortex, immunolabeling of reactive astrocytes was also noted. The cells were deemed astrocytes based on their size, morphology, and distribution within the laminated regions of the hippocampus. The α7 immunopositive astrocytes seemed to cluster around amyloid deposits, which was confirmed when brain sections were counter stained with Congo red (Fig. 2). This micrograph shows mAb 319 positive cells associated with a Congophilic plaque. APP mice showed similar patterns of astrocytic staining, but to a lesser degree, possibly due to the lower amyloid burden in these mice (data not shown). PS1 mice resembled nontransgenics; rarely were α7 immunolabeled astrocytes seen in those mice.
Figure 2. Reactive astrocytes co-localize with Congophilic plaques in APP+PS1 mice.

Horizontal sections were immunostained using mAb 319 at a 1:5000 dilution, and then counterstained with Congo red. Micrographs show the molecular layer of the CA3 region of the hippocampus. Black staining of the cell bodies is clearly defined, with faint staining of processes, all surrounding the red plaque core. Scale bar = 50 µm.
To determine if α7 expression by reactive astrocytes was due to a generalized inflammatory response to amyloid, we injected LPS into the hippocampus of APP and nontransgenic mice. LPS treatment resulted in a diffuse pattern of mAb 319 immunolabeling of astrocytes, cells known to react to LPS injections (Hauss-Wegrzyniak et al., 1998). No changes were seen in neuronal staining with mAb 319 (Fig. 3). Shown are the CA3 regions of saline injected (panels A,C) versus LPS injected mice (panels B,D). A few immunopositive astrocytes were seen in saline treated APP mice (panel C), but not in the nontransgenics (panel A). Both nontransgenics (Fig. 3B) and APP (Fig. 3D) mice showed reactive α7 immunopositive astrocytes upon LPS treatment.

Figure 3. LPS stimulates astrogliosis and immunolabeling by anti-α7 antibodies.
Nontransgenic (A,B) and APP (C,D) mice were injected with saline (A,C) or LPS (B,D) 7 days prior to sacrifice. Horizontal sections were immunostained using mAb 319 at a 1:5000 dilution. Micrographs show the CA3 region of the injected hippocampus. Pyramidal cells were intensely stained, located in the upper left corner of each panel. LPS treatment resulted in widespread activation of astrocytes which were immunolabeled by mAb 319 (B,D). Scale bar = 50 μm. Abbreviations: NT = nontransgenic, APP = mutant amyloid precursor protein transgenic, LPS = lipopolysaccharide.
Figure 3. LPS stimulates astrogliosis and immunolabeling by anti-α7 antibodies.
In a final set of experiments, LPS was injected into α7 null mice, thus testing the specificity of the anti-α7 antibodies. Mice carrying a null deletion for α7 were genotyped and selected to be either nontransgenic (α7+) or homozygous null (α7-). Genotyping results indicated a single band at 440 bp (α7+) or 750 bp (α7-). Figure 4A shows typical PCR product banding for nontransgenic and null genotypes. All mice were genotyped and sorted based on these results. Subsequently, RNA was extracted from the hippocampus, reverse transcribed, and subjected to PCR to determine whether α7 mRNA was expressed. Mice that had been genotyped as α7-, did not express α7 mRNA. Typical samples are shown in Figure 4B, with a positive signal at 381 bp in lane 1 (nontransgenic), as well as a smaller primer-dimer product running at the end of the gel (present even in the absence of cDNA). All animals genotypically α7+ also had α7 mRNA; none of the α7 null mutant mice had α7 mRNA.
Figure 4. Genotyping and RNA analysis of $\alpha^7+$ and $\alpha^7-$ mice. Panel A shows a typical PCR product with ethidium bromide/UV detection of genomic DNA. Lanes 1-2 are from an $\alpha^7+$ mouse, showing only a 440 bp band in lane 1 indicating the wild type allele, but no band for the neo-cassette primers in lane 2. Lanes 3-4 are from a homozygous null mutant $\alpha^7-$ mouse, lacking the band for the wild type primers in lane 3, showing only a 750 bp band indicating the neo-cassette disrupted allele in lane 4. Panel B shows a typical RT-PCR product derived from mRNA and detected with ethidium bromide/UV. Both lanes show a small product due to primer-dimer formation. Lane 1 shows a single specific band of the expected size of 381 bp from an $\alpha^7+$ mouse. Lane 2, from a homozygous null mutant $\alpha^7-$ mouse, shows no specific bands.
Immunohistochemistry was performed using tissue from both α7+ and α7- mice, with or without LPS injection. Three different commercially available antibodies were used (Table 1). Shown in Figure 5 are three anti-α7 antibodies (mAb 319 in panels A-B; mAb 306 in panels C-D; pAb H-302 in panels E-F). Each antibody showed intense, ubiquitous labeling of neurons throughout the brain; cortical neurons are shown in this figure. No difference in the staining patterns was seen between untreated α7+ (Panels A,C,E) and α7- mice (Panels B,D,F). All three antibodies immunolabeled neurons in both genotypes. LPS injection into the hippocampus caused reactive astrogliosis similar to that seen in Figure 3 (data not shown). Again, mAb 319 labeled astrocytes in both α7+ and α7- mice. Astrocytes were not labeled by mAb 306 nor pAb H-302 in either genotype or treatment. Thus, regardless of treatment or antibody used, both α7+ and α7- mice showed distinct nonspecific labeling of neurons (mAbs 306, 319, pAb H-302) as well as astrocytes (mAb 319).
Figure 5. Immunohistochemical analysis of α7+ and α7- tissue. Horizontal sections were immunohistochemically analyzed using various anti-α7 antibodies. Micrographs show neurons in the frontal cortex. Ubiquitous staining of cell bodies was seen with all antibodies. Some cytoplasm and processes are stained with mAb 306 (C-D, 1:3000 dilution) and pAb H-302 (E-F, 1:200 dilution). mAb 319 primarily stained neuronal cell bodies and nuclei (A-B, 1:5000 dilution). Neurons in both α7+ (A,C,E) and α7- tissue (B,D,F) were immunolabeled by these anti-α7 antibodies. Scale bar = 50 μm.
Antibody staining patterns were also examined by Western blot, using cortical tissue from both genotypes, with or without LPS injection. The exposure times were increased to maximize detection of faint bands. Although most antibodies showed many nonspecific bands, no antibody revealed differences in protein bands in untreated $\alpha^{7+}$ versus $\alpha^{7-}$ mice at the expected molecular weight of 56 kDa (Fig. 6). LPS injection caused upregulation of an unknown protein in both $\alpha^{7+}$ and $\alpha^{7-}$ mice at approximately 45 kDa (mAb 306) and 75 kDa (mAb 306, pAb H-302). No additional bands were seen with mAb 319 after LPS treatment. However, none of the antibodies detected differences between LPS treated $\alpha^{7+}$ versus $\alpha^{7-}$ mice.
Figure 6. Western blot analysis of α7+ and α7- tissue. Cortical tissue was homogenized, subjected to SDS-PAGE, transferred and immunoblotted. ECL detection was captured with subsequent film exposure. The scanned image shown contains typical samples of alternating lanes of α7+ tissue and α7- tissue. Tissues from both untreated control mice and LPS injected mice were run for each genotype and antibody. A primary band at 45 kDa, and a secondary band at 30 kDa was seen with mAb 319 (lanes 1-4). Strong bands at 30, 50, and 75 kDa were seen with mAb 306 (lanes 5-8). Strong bands at 30, 45, 75, and 100 kDa were evident with pAb H-302 (lanes 9-12). Both nontransgenic α7+ and null mutant α7- tissue were immunolabeled by these anti-α7 antibodies. LPS treatment resulted in an increase in immunolabeling by mAb 306 (bands at 50 and 75 kDa) and pAb H-302 (bands at 75 kDa). This increase in staining was seen in both α7+ and α7- tissue.
Discussion

Three different commercially available antibodies intended to label α7 nAChR subunits were used in standard immunohistochemical and immunoblotting protocols. The antibodies were generated using different host species, and different α7 subunit sequences, though all products are directed towards portions of exon 10.

Previous reports have shown the utilization of monoclonal antibodies from clones 306 and 319 for immunohistochemical analysis of rat brain (Dominguez del Toro et al., 1994). The results reported by Dominguez del Toro indicated ubiquitous neuronal expression, similar to what we saw in our studies. Both the Dominguez del Toro procedure and ours used paraformaldehyde fixation of the brain prior to sucrose cryoprotection. Both procedures used 25 µm free floating sections, an avidin-biotin-peroxidase protocol, and DAB color development. The immunohistochemical protocols used in our experiments were developed to yield significant positive stain while minimizing background. Various concentrations and combinations of primary (1:100-1:10000) and secondary antibodies (1:1000-1:10000) were tested in an effort to identify conditions that stained the α7+/+, but not the α7 null mice, without success. Tissue perfusion (saline versus paraformaldehyde) and post fixation times (2-24 hr) were also evaluated separately in nontransgenic α7+ mice. No differences in the immunolabeling patterns were seen with any of these variations. It is conceivable that the antigenic determinant(s) was modified by the paraformaldehyde treatment (Montero, 2003). Another possibility is that the levels of α7 may be so low, or the amount of antibody needed to yield a signal so high, that cross-reacting protein binding masked any α7 positive cells. Other researchers that have worked with the α7 null mice did not use
immunohistochemistry to demonstrate the absence of this receptor, but rather showed the absence of α-bungarotoxin binding (Orr-Urtreger et al. 1997; Franceschini et al., 2002; Wang et al., 2003). Using our paraformaldehyde treated tissue, we failed to detect specific labeling using fluorophore conjugated α-bungarotoxin (data not shown).

The immunolabeling of reactive astrocytes in amyloid depositing mice by mAb 319 was an interesting finding of these studies. Subsequent studies with LPS injection in α7 knockout mice revealed this labeling to be nonspecific. Recent reports by Teaktong and colleagues (2003) showed immunolabeled α7+ astrocytes in human AD brain. Cholinergic signaling by rat astrocytes has also been demonstrated (Sharma and Vijayaraghavan, 2001). Activation of these cells produced calcium flux that was blocked by α-bungarotoxin, indicating the presence of α7 nAChRs. The potential for some of the astrocyte α7 labeling to be specific should not be ruled out. Thus, in designing experiments to evaluate α7 expression an alternative approach would be the use of α-bungarotoxin instead of anti-α7 antibodies. However, we were unable to develop adequate α-bungarotoxin labeling in formaldehyde fixed sections.

Several reports have demonstrated Western blot analysis of α7 in rodent brain extracts using monoclonal antibodies from clones 306 and 319 (Schoepfer et al., 1990; Dominguez del Toro et al., 1994; Orr-Urtreger et al., 1997; Dineley et al., 2001; Fabian-Fine et al., 2001). These reports have listed the apparent molecular weight of the α7 subunit ranging from 48-72 kDa, though the calculated molecular weight is 56 kDa. Some of the investigators used standard homogenization procedures, SDS-PAGE separation, transfer, and subsequent immunoblotting, similar to the procedure described here. Other investigators affinity purified the α7 nAChRs with cobra toxin or
bungarotoxin prior to SDS-PAGE, resulting in a primary band at approximately 56 kDa (Dominguez del Toro et al., 1994; Orr-Urtreger et al., 1997). The immunoblotting protocols used in our laboratory were developed to minimize nonspecific bands and background. Various concentrations and combinations of primary and secondary antibodies, ranging from 1:50-1:10000, were tested in an effort to come up with conditions that differentiated between the α7+ and the α7 null mice, without success. Separately, various homogenization buffers (10 mM HEPES ± 1-2% triton-x 100, 50-150 mM PBS ± 1-2% triton-x 100, 50 mM TBS ± 1-2% triton-x 100), vendors of secondary antibodies (Vector, Santa Cruz), blocking solutions (BST + 2.5-5% milk, TBST + 2.5-5% milk), and subcellular fractionation preparations (crude, membrane, and solubilized fractions) were tried, but failed to reveal differences in the staining pattern between α7+ and α7 null mice. Other researchers that have performed Western blot analysis of α7 null tissue did not use commercially-available antibodies, and also affinity purified the α7 subunit prior to analysis (Orr-Urtreger et al. 1997; Franceschini et al., 2002; Wang et al., 2003). Again, an alternative approach in designing experiments to evaluate α7 expression would be the use of α-bungarotoxin affinity purification. However, the large amounts of tissue needed for affinity purification complicate the procedure and preclude analysis of small brain regions (such as the hippocampus) in individual rodents.

There have been reports of a partial duplication of the human α7 nAChR gene, which has four novel N-terminus exons and conserved exons 5-10 (Gault et al., 1998; Villiger et al., 2002). Such duplication has not been reported in rodents, but cannot be completely ruled out. If there were such duplication, it might account for the α7 protein detection in the absence of α7 gene expression. Still, one would expect at least a
quantitative difference in the amounts of stained material. Alternatively spliced α7 mRNAs have also been shown in mice (Saragoza et al., 2003). The resultant mRNA includes a novel exon 9b. Additionally, another splice variant with novel exon 4b has been reported in rat (Severance et al., 2004). The Saragoza variant could potentially interfere with our RNA analysis, as the primers are designed to prime to exons 9-10. In contrast, the Severance variant does contain exons 9-10 and should be eliminated in the knock out mouse. However, alternatively spliced mRNAs would have no effect on the genotyping results as exons 8-10 are interrupted in the knock out. Moreover, the complete absence of α7 mRNA in the null mice would require some mutation in any duplicated α7 gene which would disrupt the primer pairs from annealing. We find such circumstances unlikely to account for the results we have obtained here.

In conclusion, careful examination of protocols will be required in order to draw any conclusions made from immunodetection studies of α7 nAChRs. Localization of the α7 subunit with immunohistochemistry must be interpreted with caution. Confirmation with α-bungarotoxin binding experiments is recommended, as well as RNA analysis where applicable.
Chapter 2
Acute and Chronic Microglial Inflammatory Responses After Intrahippocampal Administration of Lipopolysaccharide

Abstract

Inflammation has been argued to play a primary role in the pathogenesis of neurodegeneration in Alzheimer’s disease. The inflammatory response can be either beneficial or harmful, depending particularly upon the duration of the reaction. Lipopolysaccharide (LPS) activates the innate immune response and triggers gliosis when injected into the central nervous system. In studies described here, we evaluated the time course of microgliosis after acute intraparenchymal administration of LPS. Mice were injected bilaterally into the hippocampus with 4 µg of LPS. Post injection survival times were 1, 6, and 24 hr, as well as 3, 7, 14, and 28 days. Protein and RNA analyses were performed for inflammatory markers. Significant elevations of cluster differentiation marker CD45, glial fibrillary acidic protein (GFAP), scavenger receptor A (SRA), and Fcγ receptor mRNA were seen after 24 hr. Immunohistochemistry revealed a complex pattern of protein expression by microglia coupled with changes in morphology. RNA and protein for Fcγ receptor, GFAP, and SRA were transiently elevated, peaking at 3 days, and returned to basal levels after a week. In contrast, microglia remained significantly activated through the 28 day time point as determined by CD45 and complement receptor 3 levels. These findings indicated that the inflammatory response
had both an acute and chronic response to LPS that was mediated primarily by microglia in the central nervous system.

**Introduction**

Neuroinflammation is a consistent pathological event in many neurodegenerative diseases including Alzheimer’s disease (AD). AD is of particular interest due to several epidemiological studies implicating NSAID use with lower levels of AD risk (McGeer & McGeer, 1996; In ‘t Veld et al., 1999). The inflammatory response of the periphery is well characterized, but until recently, the brain was considered a privileged organ, without much of an immuno-inflammatory system. We now know that the resident macrophages of the brain, microglia, coordinate local inflammation as well as the innate immune response (Streit, 2002). The inflammatory response in AD encompasses components of the complement system, cytokines and chemokines, reactive oxygen species, prostaglandins, and a host of others (Akiyama et al., 2000).

Lipopolysaccharide (LPS) has traditionally been used to stimulate the innate immune response. LPS is a Gram-negative bacterial cell surface proteoglycan, also known as bacterial endotoxin, which triggers an inflammatory response by the host. LPS binds to circulating LPS binding protein, which subsequently transfers the LPS to membrane bound cluster differentiation (CD) marker CD14, a common receptor for bacterial components (Heumann & Roger, 2002). The CD14 protein does not contain an intracellular domain, so interaction with additional components is likely. Toll like receptor 4 (TLR4), as well as myeloid differentiation protein (MD2), form a complex through which LPS:CD14 can transduce a signal (Thomas et al., 2002). Multiple cascades are involved in the signal transduction, with nuclear factor NFκB as a key
player. Downstream gene activation triggers the production of cytokines, nitric oxide, and prostanoids leading to the cellular inflammatory response.

The role of inflammation in the brain is controversial. The phagocytic capabilities of microglia have been exploited in amyloid depositing transgenic mice, aiding in the removal of Aβ deposits during passive immunization (Wilcock et al., 2003, 2004a,b) as well as after activation of the innate immune system (DiCarlo et al. 2001, Herber et al., 2004b). However, the ability of activated glia to produce cytokines, chemokines, and other noxious products may lead to neuronal damage. Many in vitro studies have demonstrated that LPS-stimulated microglia cause neuron death (Chao et al., 1992; Kim et al., 2000; Lehnardt et al., 2002). However, only under chronic activation in vivo has LPS been shown to induce enough inflammation to cause basal forebrain neurodegeneration (Hauss-Wegrzniak et al., 2002). An exception is the acute injection of LPS into the substantia nigra, which caused cell death, possibly due to the high ratio of microglial per neuron in that brain region (Kim et al., 2000).

In our studies, we have combined traditional immunohistochemistry with quantitative real time polymerase chain reaction (qRT-PCR) to better understand the inflammatory response to LPS in the brain. We injected LPS into the hippocampus of young mice and subsequently measured mRNA and protein for markers of inflammation and phagocytosis. We demonstrate the time course of this response, and the involvement in particular of microglia.
Materials and Methods

Mouse Strains

Nontransgenic mice obtained during the breeding of our APP+PS1 transgenic mouse colony (Holcomb et al, 1998) aged 5-6 months were used in this study, with 6 animals per experimental time point, for a total of 48 mice. Animals were group-housed under a 12 hr light-dark cycle with free access to chow and water. Experimental groups were balanced regarding gender.

Intrahippocampal Injections

Mice were anesthetized using isoflurane and immobilized in a stereotaxic apparatus. One microliter injections of 4 µg/µl lipopolysaccharide (Salmonella abortus equi, Sigma, Saint Louis, MO) were delivered over a two minute period into both hippocampi (stereotaxic coordinates from bregma: -2.7 mm posterior; +/-2.5 mm lateral; -3.0 mm ventral). This procedure had been previously demonstrated in our lab to induce a neuroinflammatory response without adversely affecting animal survival or causing neurotoxicity (DiCarlo et al., 2001). The control group consisted of untreated mice. LPS treated mice survived 1, 6, 24, 72, 168, 336, or 672 hr post injection. Animals were singly housed for the post treatment survival period under standard vivarium conditions. All animal work was conducted under National Institute of Health guidelines, and approved by the University of South Florida’s institutional animal care and use committee.

Tissue Preparation

Mice were anesthetized with pentobarbital (200 mg/kg, ip), then perfused transcardially with 25 ml of saline. The brain was removed, the right hippocampus was dissected, and the tissue was then stored at –80°C for subsequent biochemical analyses.
For immunohistochemistry, left hemispheres were transferred into a 4% neutral buffered paraformaldehyde solution for 24 hr, then processed through a cryoprotection schedule of 10, 20, then 30% sucrose (24 hr in each solution). The tissue was sectioned horizontally on a sliding microtome at 25 µm. Sections were then stored in Dulbecco’s phosphate buffered saline pH 7.4 (DPBS) with sodium azide (100 mM) at 4°C.

**RNA Analysis**

Tissue from the right hippocampus was analyzed for mRNA expression via reverse transcription followed by qRT-PCR, as described by Dickey and colleagues (2003). RNA was extracted from the injected hippocampus using Qiagen’s Rneasy® procedure (Valencia, CA). The hippocampus was homogenized in 700 µl of RLT buffer for 30 seconds using an electronic rotor-stator. The manufacturer’s protocol for RNA preparation was followed as indicated, including the optional on-column DNase digestion. Sample RNA was then assayed using Molecular Probes RiboGreen® RNA quantitation kit (Molecular Probes, Eugene, OR). A series of standard RNA solutions were prepared ranging from 20-1000 ng/µl. Samples and standards were prepared in triplicate, using 1 µl of sample per 200 µl reaction volume. Fluorescence was monitored at 485 nm excitation and 538 nm emission wavelengths. Each sample RNA was then diluted in water to a final concentration of 50 ng/µl and 5 ng/µl. The 50 ng/µl samples were run on a 1% agarose gel using ethidium bromide/ultraviolet detection.

A composite of all RNA samples was made in order to prepare a standard curve for qRT-PCR analysis. A pool consisting of 2 µl of each 50 ng/µl sample was made. Serial dilutions of this composite standard were made down to 0.2 ng/µl to construct a standard curve. Reverse transcription (RT) with mMLV (Invitrogen, Carlsbad, CA) was
then performed on the standard dilutions and 5 ng of each sample RNA solution, using
the master mix and conditions detailed in Table 2. The resulting cDNA was then
subjected to qRT-PCR using SYBR® Green PCR Master Mix (Molecular Probes,
Eugene, OR). Table 3 contains the primer sequences and cycling conditions used in the
PCR reaction. The sequence for scavenger receptor B is identical to that listed for CD36
as listed in GenBank; note that the same sequence is listed for SR-B1 and various other
pseudonyms. For each reaction, 12.5 µl of SYBR Green, 7.5 µl water, 1.5 µl of each
primer, and 2 µl of the 5 ng sample dilutions were run using an MJ Research DNA
Engine™ Cycler with Opticon™ Detector (MJ Research, Inc., Waltham, MA).
Quantitation of several mRNAs was performed using the cDNA generated by reverse
transcription.
Table 2. Reverse Transcription Master Mix and Cycling Conditions

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Cycling Conditions

1) 25°C for 10 min
2) 42°C for 30 min
3) 60°C for 30 min
4) 95°C for 5 min
5) 4°C for storage
Table 3. Primer Sequences for qRT-PCR

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</table>
| Cluster differentiation marker (CD14)       | F: 5’-GGAACATTTGCATCCTCCTG-3’  
|                                             | R: 5’-TGAGTTTTCCCTTCTCCGCTT-3’                    |
| Cluster differentiation marker (CD45)       | F: 5’-CAGAGCATTTCCAGGGTTATT-3’  
|                                             | R: 5’-GGACCTGCATCTCCATTATTA-3’                    |
| Fc gamma receptor IIb (FcγRIIb)             | F: 5’-GGAAAGAAGCTGCAAAACTG-3’  
|                                             | R: 5’-CCAATGCCAGGGAGACTAA-3’                      |
| Glial fibrillary acidic protein (GFAP)       | F: 5’-GATCGCCACCTACAGGAAAT-3’  
|                                             | R: 5’-GTTTCTCGGATCTGGAGGT-3’                       |
| Interleukin 1 beta (IL1β)                   | F: 5’-CTCATTGTGGCTGTGGAGAA-3’  
|                                             | R: 5’-GCTGTCTCTAAATTGGAACGTCA-3’                   |
| Ribosomal RNA 18S subunit (18S rRNA)        | F: 5’-GTAACCCCGTTGAACCCCATT-3’  
|                                             | R: 5’-CCAATGCCAGGGAGACTAA-3’                      |
| Scavenger receptor A (SRA)                  | F: 5’-GACGCTTCCAGAATTTCCAGC-3’  
|                                             | R: 5’-ATGTCCTCTGGTTGCTTTG-3’                       |
| Scavenger receptor B (SRB)                  | F: 5’-AAGTGGTCAACCCATAACGAG-3’  
|                                             | R: 5’-ACTTGTCAAGGCTGGAAATGG-3’                     |
| Synaptotagmin                               | F: 5’-CATCGACCAGATCCACTTGT-3’  
|                                             | R: 5’-TCGTTCTCTATTTCGACAC-3’                       |
| Toll like receptor 4 (TLR4)                 | F: 5’-GCGGGAAGGTTATTGTGGTA-3’  
|                                             | R: 5’-AGGCAGTACCAATTTCACCCTG-3’                    |
| Tumor necrosis factor alpha (TNFα)          | F: 5’-CTGTGAAGGGAATGCGGTGTT-3’  
|                                             | R: 5’-CCAGCATTTGTGTTTCTG-3’                        |

Cycling Conditions

1) 95°C for 15 min
2) 95°C for 15 sec
3) 60°C for 60 sec
4) Plate read
5) Go to line 2, 39 more times
The SYBR Green reaction occurs when the dye binds to double stranded DNA. Maximum absorbance is 497 nm, with emission at 520 nm. The Opticon instrument is compatible with a 96 well format, and uses individual excitation and detection of each well. For each mRNA of interest, a melt curve analysis of the PCR product was performed after cycling was complete. Fluorescence was read in 1°C increments from 56-99°C. Primer pairs were designed to yield a single, specific product, with no evidence of primer-dimer formation using Primer 3 software from the MIT web site http://frodo.wi.mit.edu/cgi-bin/primer3/primer3. The following rules were helpful in primer design: 50% GC content, 18-30 bases long (20 optimal), identical predicted melting temperatures of both primers (optimal is 60°C), verify no self complementarity or primer-dimer formation (additional information on potential primer-dimer is available from Qiagen on the Web at http://oligos.qiagen.com/oligos/toolkit.php - no run of more than 3 bases annealing between primers), avoid 3 or more G or C bases at 3’ end of primers, and target a product size between 100-300 bp. All primers were checked for target specificity using the NCBI homepage at http://www.ncbi.nlm.nih.gov/BLAST/.

The Quantity Calculations derived by the Opticon MONITOR™ Analysis Software (version 1.07) yeild the cycle number at which the fluorescence exceeds the background level, called the C(T) value. In general, the lower the C(T) value, the higher number of cDNA copies in the sample. For each mRNA of interest, the C(T) values from the PCR were determined. To create a linear curve, the log of ng RNA in each standard was plotted against the average C(T) value (each sample and standard was analyzed in triplicate). Log ng RNA were calculated for each sample, and then converted to ng RNA. For a given reverse transcription, all targets of interest were normalized to results for 18S
rRNA for the same sample to control for variations in the starting RNA concentration and enzyme efficiency. The ratio of the ng target RNA to ng 18S rRNA was calculated, and the results subjected to one way analysis of variance (ANOVA), followed by Fisher’s protected least squares difference (PLSD) with significance taken at \( p < .05 \). The final results are presented as fold change versus control mice.

**Histology**

Immunohistochemical analysis of reactive glial markers was performed using four 25 \( \mu \)m free-floating sections spaced 300 \( \mu \)m apart through the hippocampus. Details of this procedure were originally described elsewhere (Gordon et al., 1997). Sections were first blocked for endogenous peroxidases (10% methanol, 3% hydrogen peroxide in DPBS) for 15 minutes, washed with DPBS, then permeabilized in a solution of 100 mM lysine, 0.2% triton x-100, and 4% normal goat or horse serum (Pel Freeze, Rogers, AK) in DPBS for 30 minutes. Sections were then incubated overnight in the appropriate primary antibody in DPBS and 4% serum (Table 4). The following day, sections were washed with DPBS, and then incubated in appropriate biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) at a concentration of 0.5 \( \mu \)g/ml in DPBS and 4% serum, for two hours. Sections were washed with DPBS, and then incubated for one hour in Vectastain® Elite® ABC solution (Vector Laboratories, Burlingame, CA). Sections were then washed and incubated with a solution of 0.5% nickelous ammonium sulfate hexahydrate and 0.05% diaminobenzidine in tris buffered saline for 5 minutes. Color development was achieved by the addition of 0.03% hydrogen peroxide, and incubation for an additional 5 minutes, followed by washes. Controls for nonspecific binding of the secondary antibody were performed by excluding primary antibodies. GFAP
immunostaining was performed using horseradish peroxidase-streptavidin (Vector Laboratories, Burlingame, CA) at 1 µg/ml, rather than ABC and nickel enhancement was not used. Sections were slide mounted and cover slipped, then imaged at 100x magnification, focusing on the dentate gyrus of the hippocampus. Images were then analyzed for area percent positive stain using Image-Pro® Plus software (MediaCybernetics, Silver Spring, MD). The results were subjected to one way ANOVA, followed by Fisher’s PLSD with significance taken at $p < .05$. The final results are presented as fold change versus control mice.

**Table 4. Primary Antibodies Used for Immunohistochemistry**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody Type</th>
<th>Source</th>
<th>Catalog #</th>
<th>Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>Rat monoclonal</td>
<td>Serotec</td>
<td>MCA1031G</td>
<td>1:3000</td>
</tr>
<tr>
<td>CR3 (CD11b)</td>
<td>Rat monoclonal</td>
<td>Serotec</td>
<td>MCA711</td>
<td>1:3000</td>
</tr>
<tr>
<td>FcγRII/III</td>
<td>Rat monoclonal</td>
<td>PharMingen</td>
<td>553141</td>
<td>1:3000</td>
</tr>
<tr>
<td>GFAP</td>
<td>Rat monoclonal</td>
<td>Zymed</td>
<td>13-0300</td>
<td>1:3000</td>
</tr>
<tr>
<td>TLR4</td>
<td>Rabbit polyclonal</td>
<td>Imgenex</td>
<td>IMG-579</td>
<td>1:3000</td>
</tr>
</tbody>
</table>

**Results**

LPS was injected into the hippocampus of nontransgenic mice and a time course established for both RNA and protein expression of inflammatory markers. Animal health overall was mildly affected by LPS. Some weight loss was seen, which was significant after 7 days, but returned to normal beginning at 2 weeks. Malaise was only notable at the 24 hr time point, primarily evidenced by hypo-locomotion.
Advantages of RNA analysis by qRT-PCR include accurate quantitation of several markers from very small samples and assessment of specific isoforms, even when suitable antibodies are not available for immunohistochemistry. Figure 7A shows a typical standard curve generated for Ribogreen assay of sample RNA concentrations. All samples were within the linear range of the assay, averaging 65 ng of RNA per µl of sample extract. Figure 7B shows typical ethidium bromide/UV detection of the RNA. All samples were intact, with no evidence of RNA degradation. Samples at a concentration of 5 ng/µl in water, as well as standards spanning 0.2-50 ng/µl in water, were subjected to qRT-PCR. Melt curve analysis of the PCR product indicated a single specific reaction product. An example of a typical melt curve is shown in Figure 7C. A single peak without shoulders or evidence of multiple peaks indicated specific primer annealing. The standard curves for the qRT-PCR were then generated, as shown in Figure 7D. All targets had correlation coefficients of not less than 0.95, with less than 25% variability in the slopes between the curves for different target mRNAs (for example, comparing 18S to IL1β). The slope represents the efficiency of the PCR reaction for the particular primer pair used. Less than 10% variation would be necessary to detect more subtle changes in gene expression as variations in the efficiency of the PCR of the target compared to 18s could artificially bias the result.
Figure 7. mRNA analysis by qRT-PCR. RNA from the hippocampi of LPS injected mice was extracted and assayed. Panel A shows the standard curve used for RiboGreen® estimation of total RNA levels. Panel B shows the typical ribosomal banding pattern of 50 ng total RNA samples, indicating the absence of significant degradation. After reverse transcription, cDNA was subjected to qRT-PCR. Panel C shows the melt curve for 18s rRNA qRT-PCR product, typical of all primers used. A single peak without shoulders indicated specific priming. Panel D shows a standard curve generated for the 18s rRNA qRT-PCR, plotting mass of the RNA added to the reverse transcription versus the threshold cycle C(T). All primers used in this work generated a standard curve with slopes varying by less than 25%.
A time course was established for LPS stimulated gene transcription. Figure 8A contains cell-specific markers. The glial markers CD45 and GFAP both changed their expression over time, whereas synaptotagmin did not change ($F_{(8,45)} = 25, p < .0001$; $F_{(8,45)} = 35, p < .0001$; $F_{(8,45)} = 1.2, p = .30$, respectively). Post hoc analysis revealed significant differences compared to untreated control mice, with significance taken at $p < .05$. The microglial marker CD45 was significantly elevated by 6 hr, peaked at 3 days (72 hr), and remained elevated throughout the time course to 28 days (672 hr) post injection. GFAP followed a similar time course and increased significantly from 6 hr to 7 days (168 hr), but returned to baseline by 14 days (336 hr). Figure 8B shows results for three receptors involved in phagocytosis. Scavenger receptor A (SRA), scavenger receptor B (SRB), and Fcγ receptor IIb (FcγRIIb) all changed expression over time ($F_{(8,45)} = 9.5, p < .0001$; $F_{(8,45)} = 6.4, p < .0001$; $F_{(8,45)} = 25, p < .0001$ respectively). Post hoc analysis revealed significant differences compared to untreated controls, with significance taken at $p < .05$. SRA was significantly elevated by 6 hr, peaked at 3 days, and then dramatically declined by 14 days. In contrast, SRB was briefly and modestly elevated only at the 6 hr time point, otherwise remaining unchanged. The FcγRIIb followed a similar time course to SRA, but remained modestly elevated out to 28 days.
Figure 8. *Time course of mRNA expression.* RNA from the hippocampus of mice injected with LPS and allowed to survive 1 hr, 6 hr, 24 hr, 72 hr (3 days), 168 hr (7 days), 336 hr (14 days), and 672 hr (28 days), was subjected to qRT-PCR. Results are presented as mean ± SEM of fold change compared to untreated control mice (n=6 each group). Note that the x-axis is not linear in order to permit evaluation of both early and late time points. Panel A shows the cell specific markers CD45 (microglia), GFAP (astrocytes), and synaptotagmin (neurons). Both CD45 and GFAP levels peak around 3 days; only CD45 remained elevated at 28 days. Synaptotagmin levels were unaffected by treatment. Panel B shows three receptors capable of mediating phagocytosis, SRA, SRB, and FcγRIIb. SRA was transiently elevated, peaking from 1-3 days. FcγRIIb also peaked around 3 days, but remained modestly elevated at 28 days. SRB had a small increase, significant only at 6 hr. Panels C and D show results of RNA from the hippocampus of mice injected with LPS and allowed to survive 72 hr (3 days). Panel C shows significant increases in both TLR4 and CD14 messages. Panel D shows dramatic increases in TNFα and IL1β messages; note greater than 30 fold increases of IL1β. * p < .05 for all panels versus untreated controls.
Figure 8. Time course of mRNA expression.
Based on the findings that most of the target RNAs peaked at 3 days, several additional markers were examined at that time point. Figure 8C shows the relative expression of two key components of the LPS signaling cascade, compared to untreated control mice. Both the LPS receptor cluster differentiation marker (CD14) and toll like receptor (TLR4) were significantly elevated. Figure 8D shows the relative contributions of interleukin (IL1β) and tumor necrosis factor (TNFα). IL1β message was increased by greater than 30-fold. TNFα was also significantly elevated, but to a lesser extent.

Immunohistochemical analysis of horizontal brain sections was conducted to verify the cell-specific expression of several inflammatory markers identified by RNA analysis. Figure 9 shows the time course of changes in expression for four microglial markers. Representative micrographs from the site of LPS injection (the hilus of the dentate gyrus subregion of the hippocampus) are presented. Untreated mice demonstrated resting, ramified microglia, with fine delicate processes which were marked by CD45 and complement receptor CR3 (Fig. 9A-D). LPS treatment resulted in a widespread activation of microglia with a concurrent shift in morphology. After 1 hr (Fig. 9E-H), cells increased expression of CD45, CR3, and Fcγ receptor II/III (FcγRII/III) and developed thicker, shorter processes; this increase was more obvious at 6 hr (Fig. 9I-L). By 24 hr, the increases were observable for all four markers CD45, CR3, SRA, and FcγRII/III (Fig. 9M-P). The ramified microglia predominated at this time point, but small, rounded cells were also seen. After 72 hr, the staining intensity was at its peak for all four markers. Multiple morphologically distinct cells were visible including hyper-ramified (bushy, indicated by arrow in Fig. 9S), round, and amorphous/ameboid cells with smaller numbers of thick processes (Fig. 9Q-T). After a week, SRA and FcγRII/III
expression had subsided, but CD45 and CR3 remained high. The bushy microglial morphology predominated (Fig. 9U-X). An interesting finding was both CD45 and CR3 continued to mark bushy, reactive microglial after 2 weeks (Fig. 9Y-BB) and 4 weeks (Fig. 9CC-FF).

Figure 9. Time course of microglial protein expression and morphology. Brains from LPS injected mice were sectioned horizontally at 25 µm and immunostained. Each row represents a different time point: untreated mice (A-D); LPS treated mice (E-FF) survived 1 hr (E-H), 6 hr (I-L), 24 hr (M-P), 3 days (Q-T), 7 days (U-X), 14 days (Y-BB), and 28 days (CC-FF). Each column represents a different marker: the first column is CD45, the second column is CR3, the third column is SRA, and the fourth column is FcyRII/III. Micrographs are from the hilus of the dentate gyrus of the hippocampus. Resting microglia are seen after saline treatment (A-D), expressing CD45 (A) and CR3 (B). From 1 hr (E-H) to 6 hr (I-L) cells became swollen and increased levels of CD45 (E,I), CR3 (F, J) and FcyRII/III (H,L) are apparent. By 24 hr (M-P), all four markers are expressed, on both round and ramified cells. At 3 days, expression levels are peaking, as round, ameboid, and ramified/bushy cells stain intensely for CD45 (Q), CR3 (R), SRA (S), and FcyRII/III (T). The arrow in Panel S indicates a bushy morphology. At 7 days (U-X), 14 days (Y-BB), and 28 days (CC-FF), SRA (W, AA, EE) and FcyRII/III (X,BB,FF) decrease to control levels; CD45 (U,Y,CC) and CR3 (V, Z, DD) remained elevated. Scale bar = 50 µm.
Figure 9: Time course of microglial protein expression and morphology.
Semi-quantitative results for microglial immunohistochemical analyses in the dentate gyrus are shown in Figure 10. CD45, CR3, SRA, and Fc\(_\gamma\)RII/III all changed significantly over time \(F_{(8,45)} = 11.4, p < .0001\) \(F_{(8,45)} = 12.3, p < .0001\) \(F_{(8,45)} = 3.4, p = .0037\) \(F_{(8,45)} = 4.4, p = .0006\), respectively. Post hoc analysis revealed significant differences compared to untreated control mice, with significance taken at \(p < .05\).

Results are expressed as fold change versus untreated controls. Up to three fold increases in CD45 levels were seen, beginning at 1 hr and lasting to 28 days (Fig. 10A). A similar profile for CR3 was obtained, with up to four-fold increases from 1-28 days (Fig. 10B). SRA, though significantly increased only at 1 and 3 days, showed over 30 fold change relative to the virtual absence of staining in control animals (Fig. 10C). Fc\(_\gamma\)RII/III staining was also transiently and significantly elevated at 1 and 3 days by 25 fold (Fig. 10D).

**Figure 10. Quantitation of immunohistochemical results for microglial markers.** Brains from LPS injected mice were sectioned horizontally at 25 µm, and immunostained. Images were collected from the dentate gyrus of the hippocampus at 100x magnification. The percent area occupied by positive stain was calculated. Results shown are fold change compared to uninjected mice. Note that the x-axis is not linear in order to permit evaluation of both early and late time points. CD45 was significantly elevated from 1 hr out to 28 days. CR3 displayed a similar time course, with significant increases from 1-28 days. In contrast SRA and Fc\(_\gamma\)RII/III were transiently activated, with significant increases only at 1-3 days. *\(p < .05\).
Figure 10. Quantitation of immunohistochemical results for microglial markers.
Astrogliosis was also evaluated in response to LPS and is shown in Figure 11. Untreated mice showed detectable levels of GFAP in the hippocampus (Fig. 11A). Three days after treatment, astrocytes throughout the injected hippocampus increase GFAP expression and had thickened processes (Fig. 11B). Figure 11C shows the dentate gyrus results expressed as fold change of stained area compared to untreated controls. A main effect of time was determined ($F_{(8,45)} = 8.2, p < .0001$). Post hoc analysis revealed significant increases beginning at 1 day, peaking from 3-7 days, and remaining modestly elevated at 28 days.

*Figure 11. Time course of astrogliosis in response to LPS.* GFAP levels in the brains of LPS injected mice were demonstrated by immunohistochemistry. Panel A depicts the hippocampus of an untreated mouse showing low, basal levels of GFAP. Panel B shows the heightened astrocyte reaction 3 days after LPS injection. Statistical analysis of the dentate gyrus subregion, shown in Panel C, revealed significant activation beginning at 1 day, peaking from 3-7 days, then remaining moderately elevated out to 28 days. The x-axis is nonlinear to show the changes at early time points.

Scale bar = 500 µm.  *p < .05.*
Figure 11. Time course of astrogliosis in response to LPS.
Finally, Figure 12 presents the immunohistochemical results for TLR4. Representative micrographs of the dentate gyrus subregion of the hippocampus are shown. No cellular staining for TLR4 was detectable in untreated animals (Fig. 12A). As early as 1 hr after injection, TLR4 immunostaining was evident as a band of staining along the inner extent of the granule cell layer of the dentate gyrus (Fig. 12B) as well as along the edge of the hippocampal fissure (not shown). Beginning at 24 hr, punctuate staining was evident in the hilus (Fig. 12C). Figure 12D shows the dentate gyrus fold change of stained area compared to controls. Post hoc analysis revealed significant increases from 1-6 hr. This is due to the intense layer of stain seen in Figure 12B. This layer of stain decreases over the first week, as punctuate stain starts to appear. The increase in the punctuate stain, though visually discernible, was not statistically significant using the area measurement ($p = .055$ at 3 days; $p = .077$ at 7 days).

*Figure 12. Time course of TLR4 levels after LPS injection.* Immunohistochemical analysis of TLR4 was conducted using 25µm horizontal brain sections from LPS treated mice. Micrographs are representative of the dentate gyrus subregion of the hippocampus. Panel A shows an untreated mouse with little to no positive stain. Panel B shows an intense layer of stain along the inner dentate granule cell layer, 1 hr after LPS injection. Panel C shows punctate and more dispersed stain in the hilus, beginning 3 days after injection. Statistical analysis, shown in Panel D, revealed significant increases at 1 and 6 hr, as well as smaller increases at 3 and 7 days. The x-axis is nonlinear to show the changes at early time points. Scale bar = 200 µm. * $p < .05$. 

58
Figure 12. Time course of TLR4 levels after LPS injection.
Discussion

Lipopolysaccharide is a prototypical inflammatory agent used in both the periphery and in the brain. Here, we describe the time course of the microglial reaction to LPS in vivo. RNA and protein data concur that there is an acute and chronic response to a single intrahippocampal injection of LPS.

The acute reaction to LPS began as early as 1 hr post injection. This short term response peaked at 3 days for both RNA and protein levels, returning to basal levels by about 7 days for several markers. The response of astrocytes was demonstrated by increases in GFAP. Several markers of the microglial response were also evaluated. Two key receptors that can mediate phagocytosis, FcγRII/III and SRA, were only briefly up-regulated, perhaps priming the system for either mounting an acquired immune response or continuing the innate response. The endogenous ligand for Fcγ receptor is typically the Fc region of IgG. However, there is no evidence in the current studies that anti-LPS antibodies were produced in the LPS-injected mice, although such a possibility cannot be ruled out. On the other hand, it has been demonstrated that LPS can induce Fcγ expression in the absence of IgG, as part of cell activation. In addition, Fc receptor expression by macrophages can be regulated by cytokines as well as by LPS (Amigorena et al., 1989; Lynch et al., 1990; Laszlo & Dickler, 1990; Loughlin et al., 1992; Keller et al., 1994). Thus, it is likely that the induction of Fcγ reflects another facet of microglial activation and not the presence of specific immunoglobulin.

Scavenger receptors play a key role in mediating innate immunity. They are pattern recognition receptors, with bacterial and viral products as typical ligands,
including LPS. It is interesting that fibrillar Aβ is also a ligand for SRA and SRB (Huseman et al., 2002). Our lab has previously shown decreased levels of Aβ after intrahippocampal injection of LPS in amyloid depositing mice (Chapter 3 of this dissertation, Herber et al., 2004b; DiCarlo et al, 2001). It is possible that LPS increased expression of SRA which promoted the removal of Aβ via phagocytosis in that study. We were surprised that we did not see large increases in SRB in the current study, which has been implicated in Alzheimer’s disease (Bamberger et al., 2003). We only detected a 33% increase in mRNA, and only at the 6 hr time point. Appropriate antibodies were unavailable for immunohistochemical analysis, thus no protein data were collected.

The chronic response to LPS was seen mainly in microglia, as demonstrated by both RNA and protein expression of CD45. This was further confirmed by CR3 immunohistochemistry. Our data showed significant microgliosis 28 days after a single injection of LPS. This confirms our earlier report using a similar protocol in amyloid depositing transgenic mice (Herber et al., 2004b). Our results from the direct injection of LPS into the central nervous system are in contrast to L5 nerve transection, where CR3 expression peaked at 14 days, but returned to baseline by 28 days (Tanga et al., 2004). Tanga and colleagues also showed a sustained response by GFAP to both nerve transection and to peripheral administration of LPS (Tanga et al., 2004; Raghavendra et al., 2004). In our model, the GFAP response was largely resolved after 7 days.

CD45 is a protein tyrosine phosphatase (reviewed in Irie-Sasaki et al., 2003). It is thought that activation of CD45 leads to dephosphorylation of key proteins, allowing for a quick response upon subsequent stimulation. CR3 is an integrin that mediates cell migration, adhesion, and phagocytosis (reviewed in Ehlers, 2000). CR3 can also bind
LPS and is involved with CD14 in the activation of NFκB. The sustained elevation of CD45 and CR3 by microglia may reflect a hyper-reactive system, remaining primed for subsequent stimuli. These same cells may also continue to produce cytokines and chemokines, creating a potentially hostile environment.

Changes in microglial morphology and protein expression after LPS injection were complex. Early in the time course (1-6 hr), we saw thickening and shortening of the cell processes as CD45, CR3 and Fcγ receptor expression increased. By 24 hr, SRA expression was also seen on these bushy, hypertrophied cells. Small round cells expressing CD45 and CR3 appeared at 24 hr, and an amorphous/ameboid cell form was at 3 days. The three cell shapes, bushy, round, and ameboid coexisted at the 3 day time point and expressed all four markers. Only the bushy phenotype persisted and only in cells expressing CD45 and CR3. The significance of these cell shapes and their contribution to cell function has yet to be determined. It is possible that the small round cells seen at 24 hr are actually peripheral monocytes that had entered the CNS after injection. Montero-Menei and colleagues showed that as much as 80% of OX-42 (rat equivalent of CR3) and ED1 (CD68) positive cells present in the rat brain 24 hr after intracerebral LPS injection were attributable to recruited monocytes (1996). Similar MHCII positive round cells have been reported 48 hr after intraventricular LPS injection in rats (Hauss-Wegrzyniak et al., 1998a,b). This same report showed MHCII positive “bushy” cells 4-7 days post injection. It is impossible to differentiate between brain and peripheral macrophages using current labeling techniques. One is tempted to assume the round and/or ameboid cells are phagocytic. Our own results in amyloid depositing
transgenic mice, showing decreased Aβ burden 3-14 days after LPS injection, suggests phagocytosis by some cell population (Herber et al., 2004b).

Several additional markers of inflammation were investigated. Increases in the RNA for cytokines IL1β and TNFα were expected after LPS administration (Kim et al., 2000; Nadeau & Rivest, 2002; Raghavendra et al., 2004). These cytokines not only prime the system but can also create a damaging environment. Neurodegeneration was not detectable in our model using cresyl violet or fluorojade stains (data not shown). Our observations of the level of inflammation in the brain indicated a spreading wave of microgliosis, using CR3 as a marker. Untreated mice showed low levels of CR3 even in resting microglia. However, at 1 hr, the entire hippocampus showed increased microgliosis. By 6 hr, the hippocampal fimbria, rhinal cortices, thalamus, brainstem, and posterior striatum were similarly affected. At 24 hr, the entire brain, including temporal, parietal, frontal, and occipital cortices showed microgliosis. This continued through 72 hr with a marked increase in the striatum. By 168 hr (7 days), the effect began to wane and only areas very near the hippocampus showed gliosis (striatum, fimbria, rhinal cortices). By 28 days, only the hippocampus was still experiencing microgliosis. Note that the very bushy, round, and amorphous/ameboid microglia described here were only seen in the hippocampus. Outside that region, microglia increased CR3 expression, but did not have marked changes in morphology. Thus, although a widespread inflammatory response was mounted in response to LPS, the neurons survived.

Here we report that both TLR4 and CD14 mRNA are induced following LPS injection. TLR4 mRNA has been localized by others in mouse microglial cell cultures, but not in astrocytes, oligodendroglia, or neurons (Lehnardt et al., 2002, 2003). In
contrast, *in situ* hybridization of rat brain demonstrated basal TLR4 and CD14 mRNA expression in the circumventricular organs, leptomeninges, and choroids plexi (Laflamme & Rivest, 2001). Instrastriatal LPS injection similarly increased CD14 mRNA, but not TLR4 as detected by in situ hybridization (Nadeau & Rivest, 2002). Immunohistochemical analysis of TLR4 levels in the central nervous system (with or without LPS) has not been reported to our knowledge. Singh and Jiang recently reported that peripheral administration of LPS did not cross the blood brain barrier, but rather bound endothelial cells which then signaled microglial to respond (Singh & Jiang, 2004). Although LPS clearly increased TLR4 RNA and immunostaining, the TLR4 immunohistochemical analysis did not reveal a population of highly ramified, bushy, reactive microglia similar to those stained by CD45. Possibly, the staining pattern we saw was endothelial cells, especially at the early time points. Conversely, the TLR4 staining pattern at 72 hr was reminiscent of the round and ameboid cells seen with CD45 staining at 72 hr. The ability of microglial to express TLR4 in response to LPS *in vivo* remains to be adequately demonstrated by dual label immunocytochemistry.

In conclusion, we have examined the time course of the response of the brain to a single intrahippocampal injection of LPS. The response had both a transient, acute phase as well as a chronic phase associated with the induced inflammation. Evaluation of the phenotype of reactive microglia may lead to a better understanding of neuroinflammatory diseases.
Chapter 3

Time-Dependent Reduction in Aβ Levels After Intracranial LPS Administration in APP Transgenic Mice

Abstract

Inflammation has been argued to play a primary role in the pathogenesis of Alzheimer’s disease. Lipopolysaccharide (LPS) activates the innate immune system, triggering gliosis and inflammation when injected in the central nervous system. In studies described here, APP transgenic mice were injected intrahippocampally with 4 or 10 µg of LPS and evaluated 1, 3, 7, 14, or 28 days later. Aβ load was significantly reduced at 3, 7, and 14 days, but quickly returned near baseline 28 days after the injection. No effects of LPS on Congophilic amyloid deposits could be detected. LPS also activated both microglia and astrocytes in a time-dependent manner. The GFAP astrocyte reaction and the Fcγ receptor microglial reaction peaked at 7 days after LPS injection, returning to baseline by 2 weeks post injection. When stained for CD45, microglial activation was detected at all time points, although the morphology of these cells transitioned from an ameboid to a ramified and bushy appearance between 7 and 14 days post injection. These results indicate that activation of brain glia can rapidly and transiently clear diffuse Aβ deposits, but has no effect on compacted fibrillar amyloid.
Introduction

Chronic neuroinflammation is a hallmark of many neurodegenerative diseases, including Alzheimer’s disease (AD, Akiyama et al., 2000). The role of inflammation in AD is controversial, with two sides to the response. Acute inflammation can be neuroprotective, aiding in removal of pathogens and protecting cells (reviewed in Streit, 2002). Conversely, excessive, chronic inflammation is thought to enhance cell death via an autotoxic mechanism (McGeer & McGeer, 2002). Current transgenic animal models replicate the amyloid pathology, glial response, and cognitive impairment of AD, without apparent neurodegeneration. One approach to more accurately reflect the conditions in AD brain is to inject lipopolysaccharide (LPS) into transgenic mice to enhance the inflammatory state of the brain. It was hypothesized that LPS dosing might also alter amyloid deposition while triggering neurotoxicity.

LPS is a Gram-negative bacterial cell surface proteoglycan, also known as bacterial endotoxin, which triggers an inflammatory response by the host. LPS binds to circulating LPS binding protein, which subsequently transfers the LPS to the cell membrane bound cluster differentiation marker CD14, a common receptor for bacterial components (Heumann & Roger, 2002). The CD14 protein does not contain an intracellular domain, so interaction with additional components is likely. Toll like receptor 4 (TLR4), as well as myeloid differentiation protein MD2, form a complex through which LPS:CD14 can transduce a signal (Thomas et al., 2002). Multiple cascades are involved in the signal transduction, with nuclear factor NFκB as a key player. Downstream gene activation triggers the production of cytokines, nitric oxide, and prostanoids leading to the cellular inflammatory response.
LPS has been used to mimic the inflammatory conditions seen in human AD brain. Acute and chronic application of LPS into the brain ventricles of rats resulted in gliosis, cytokine production, increased APP levels, cognitive deficits, and neurotoxicity limited to the forebrain cholinergic nuclei (Hauss-Wegrzyniak et al., 1998a,b, 2000, 2002; Willard et al., 1999). LPS has also been administered to amyloid-depositing transgenic mice to cause glial activation. However, some investigators reported increased Aβ levels in response to LPS (Sly et al., 2001; Qaio et al., 2001; Sheng et al., 2003), while others observed Aβ clearance (DiCarlo et al., 2001; Quinn et al., 2003). The experimental conditions of these previous reports vary, including the type and age of transgenic mice used; type, dose and route of administration of LPS; and post injection survival time.

A potential mechanism of Aβ removal includes phagocytosis. *In vitro* studies show that both microglia and astrocytes react to LPS with phagocytosis, as well as with production of potentially cytotoxic agents such as prostaglandins, cytokines, and reactive oxygen species (Kalmar et al., 2001). Thus, this model of neuroinflammation may be useful in triggering the innate immune system to clear Aβ deposits, and/or kill neurons. The experiments described herein will address the overall hypothesis that neuroglia play a key role in the inflammation seen in AD, and may represent an innate mechanism of Aβ removal. In an effort to clarify the mechanism of Aβ clearance, dose-response and time-dependence experiments were conducted using intrahippocampal injections of LPS into aged APP mice.
Materials and Methods

Mouse Strains

Transgenic mice were bred to develop Alzheimer’s like pathology using Tg2576 APP mice as described previously (Holcomb et al., 1998). APP mice and their nontransgenic littermates aged 16-17 months were used in this study. Animals were group housed prior to surgery under a 12 hr light-dark cycle with free access to chow and water.

Intrahippocampal Injections

Mice were anesthetized using isoflurane and immobilized in a stereotaxic apparatus. A single, one microliter injection of either saline, 4 µg/µl LPS, or 10 µg/µl LPS (Salmonella abortus equi, Sigma, St. Louis, MO) was delivered over a two minute period into the right hippocampus (coordinates from bregma: –2.7 mm posterior, -2.5 mm lateral, and -3.0 mm ventral). The incision was closed with wound clips, isoflurane was discontinued, and the animal revived on a heated pad. All mice completely recovered within five minutes. Animals were singly housed for the post treatment survival period under standard vivarium conditions. We used at least four mice (4-7) for each condition, balanced for gender. The dose response study had three conditions: APP mice injected with either saline, 4 µg LPS, or 10 µg LPS; all survived 7 days. The time course utilized two genotypes for a total of 10 conditions. Nontransgenics were injected with saline and survived 7 days, or were injected with 4 µg of LPS and survived 1, 3, or 7 days. APP mice were injected with saline and survived 7 days, or were injected with 4 µg of LPS and survived 1, 3, 7, 14, or 28 days.
**Tissue Preparation**

Mice were anesthetized with pentobarbital, and then perfused transcardially with 25 ml of normal saline, followed by 50 ml of freshly prepared neutral buffered 4% paraformaldehyde. The brain was postfixed in 4% paraformaldehyde solution for 24 hr, then processed through a cryoprotection schedule of 10, 20, then 30% sucrose. Frozen brains were sectioned horizontally on a sliding microtome at 25 μm. Sections were then stored in Dulbecco’s phosphate buffered saline pH 7.4 (DPBS) with 100 mM sodium azide at 4°C.

**Histology**

Immunohistochemical analysis was performed for each marker using six, 25 μm free-floating sections spaced 300 μm apart through the hippocampus. Details of this procedure are described elsewhere (Gordon et al., 1997). Briefly, sections were blocked for endogenous peroxidases (10% methanol and 3% hydrogen peroxide in 80% DPBS), washed with DPBS, then permeabilized (100 mM lysine, 0.2% triton x-100, 4% normal serum in DPBS). Sections were then incubated overnight in the appropriate primary antibody (Table 5). The following day, sections were washed, and then incubated in appropriate biotinylated secondary antibody. After another cycle of washes, the tissue was incubated with Vectastain® Elite® ABC kit (Vector Laboratories, Burlingame, CA). In the case of glial fibrillary acidic protein (GFAP), streptavidin-peroxidase was used rather than ABC. The tissue was then washed and stained with a diaminobenzidine: peroxide system, followed by final washes. In the case of CD45 and Fc gamma receptor II/III (FcγRII/III), nickel enhancement of the color development was used. The extent of nonspecific binding was assessed in the absence of primary antibodies for all assays.
Sections were mounted onto slides, dehydrated, and cover slipped with DPX (E.M. Sciences, Fort Washington, PA). Separately, compact plaques were evaluated after sections were slide mounted, by incubating in alkaline alcoholic saturated sodium chloride (AASSC), followed by 0.2% Congo red in AASSC. Tissue damage was evaluated using a 0.05% cresyl violet (pH 3.3) solution.

Table 5. Primary Antibodies Used for Immunohistochemistry

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Data Analysis

Stained sections were imaged at 100x magnification, focusing on the molecular layer of CA1, CA3, and dentate gyrus (DG) regions of the injected hippocampus. Images were then analyzed for area percent positive stain using Image-Pro® Plus software (MediaCybernetics, Silver Spring, MD). Area percent stained was calculated for each individual region of the hippocampus, as well as an average of the three regions. Results were averaged for each animal, and the treatment groups compared by one way analysis of variance (ANOVA) followed by post hoc analysis using Fisher’s protected least squares difference (PLSD, significance at $p < .05$). Results shown are for the CA3
region, which showed the greatest effect. Similar results were seen in all of the hippocampal regions analyzed, as well as for the average of the three.

Results

Dose Response

Intrahippocampal injections of LPS were made into APP transgenic mice in order to optimize the Aβ reductions reported previously (DiCarlo et al., 2001). Dose response studies were done using saline, 4, or 10 µg/µl of LPS. Animals were evaluated 7 days after the injection by immunohistochemistry for total Aβ burden using an anti-Aβ antibody which recognizes both Aβ1-40 and Aβ1-42. Micrographs represent the injected hippocampus of a saline injected control (Fig. 13A) versus a 10 µg LPS-dosed APP mouse (Fig. 13B) are shown. In the control mouse, both lightly stained, diffuse Aβ deposits and darkly stained, compacted deposits were seen. LPS injection resulted in removal of a major portion of the diffuse deposits in the CA1 and CA3 regions of the hippocampus. The quantification are from the CA3 region (the region indicated by the box in Fig. 13D). Statistical analysis of the data shown in Figure 13C indicated a significant removal of Aβ in LPS-injected APP mice ($F_{(2,13)} = 6.6, p < .05$).

The effect of LPS injection on compact amyloid plaque burden in APP mice was determined with Congo red staining. Compact plaques were detected typically near the hippocampal fissure in the saline injected mice (Fig. 13D). No detectable changes in the amyloid load were seen with LPS treatment in any hippocampal subfield (Fig. 13E). Quantitation also failed to reveal a significant change in amyloid burden in CA3
(Fig. 13F), CA1, or dentate gyrus subfields, or in the average of all fields from the hippocampus. Similarly, LPS treatment was not accompanied by reductions in amyloid detected by thioflavin-S (results not shown).

Figure 13. Dose response of LPS stimulated Aβ removal. APP mice were injected with saline (A,D) or 10 µg of LPS (B,E) 7 days prior to sacrifice. Immunostaining for total Aβ burden is shown in panels A and B. A significant reduction at both dosages is shown in panel C. Histochemical staining of compact plaques with Congo red dye is shown in panels D and E. No reduction was detected as a result of LPS dosing (F). The CA3 subregion enclosed by a box in Panel D represents the area used for calculating percent positive stain in data analyses (C,F). Abbreviations: CA1, CA3: subregions of Ammon’s horn; DG: dentate gyrus. Scale bar = 250 µm. * p < .05.
Gliosis, a possible link to the mechanism of Aβ reduction in APP mice, was analyzed using CD45 staining for reactive microglia. Saline-injected APP mice (controls) are shown in Figure 14A. These mice have CD45+ microglia largely restricted to the vicinity of putative amyloid plaques and the injection site within the hilus of the dentate gyrus. We use the term “putative” amyloid plaques because, even though such aggregates of microglia have previously been demonstrated to surround Congophilic cores (Gordon et al.; 2002), no Congo red counterstain was performed on these sections. LPS treatment of APP mice caused a widespread eruption in the number and staining intensity of CD45+ microglia throughout the hippocampus (Fig. 14B). Both doses caused significant increases in CD45 immunoreactivity (mean area % for saline dose = 1.1 ± 0.3; 4 µg dose = 12.3 ± 4.0; 10 µg dose = 9.1 ± 2.6). The 10 µg dose caused increased reactivity to extend outside the injected hippocampus, spreading into the adjacent cortices and even to the contralateral hippocampus (data not shown).

Astrogliosis was detected using the astrocyte specific marker GFAP. Saline-injected APP mice had diffuse distribution of GFAP+ astroglial cells, as well as plaque-associated clusters of astroglia (Fig. 14C). LPS treatment caused an increase in the staining intensity of GFAP staining throughout the hippocampus (Fig. 14D). Both doses caused a mild, but not significant, increase in GFAP immunoreactivity, (mean area % for saline dose = 17.1 ± 4.2; 4 µg dose = 19.7 ± 3.9; 10 µg dose = 18.6 ± 3.1). The highest dose triggered a notable glial response outside the injected area (data not shown).
Figure 14. Glial response to LPS. APP mice injected with saline or with 10 µg of LPS, 7 days prior to sacrifice, showed increased microglial activity due to LPS (B) versus saline (A). GFAP immunoreactivity showed increased astrocyte activity in LPS- injected (D) versus saline- injected (C) APP mice. Scale bar = 250 µm.
The potential for LPS injection to cause tissue damage was examined histochemically with cresyl violet stain. Sections were evaluated for evidence of neurodegeneration, which included pyknotic nuclei as well as gross cell loss in the pyramidal and granule cell layers of the hippocampus. Some animals had evidence of mechanical trauma in the injection vicinity, but this was observed in both saline- and LPS-injected mice to the same degree. No widespread evidence of neuronal damage caused by LPS was detected.

Overall, the dose response study demonstrated that LPS injection into the hippocampus of APP mice led to clearance of Aβ within one week, without altering compact plaque load or triggering neurodegeneration. Concurrently, treatment enhanced gliosis, suggesting a glial-mediated mechanism of Aβ reduction. The 4 µg LPS dose was chosen for use in subsequent experiments as adequate clearance of Aβ was seen at that dose.

**Time Course**

To further evaluate the LPS-induced clearance of Aβ, a time course was conducted. APP mice were injected with 4 µg of LPS, and allowed to survive 1, 3, 7, 14, or 28 days. Nontransgenic mice were also injected with LPS, and survived 1, 3, or 7 days post injection. Control groups for both genotypes received saline injections, with 7 day survival periods. Aβ burden in APP mice was determined immunohistochemically using an anti-Aβ antibody, and analyzed in the CA1, CA3, and DG subfields of the hippocampus. Analysis of variance showed a time-dependent effect on Aβ levels in LPS-injected mice ($F_{(5,30)} = 2.7, p < .05$), particularly in the CA3 subregion (Fig. 15). Post hoc analysis using Fisher’s PLSD indicated a statistically significant removal of Aβ in LPS
injected APP mice from 3-14 days, which returned to baseline by 28 days. As in the dose response study at 7 days, no effect of LPS injection on Congo red stained plaques in APP mice was seen in any subregion of the hippocampus, or in the hippocampus overall, at any time point (data not shown).

Figure 15. Time dependent removal of Aβ by LPS injections. APP mice were injected with saline or 4 µg of LPS and immunostained for Aβ. Mice injected with LPS were killed at 1, 3, 7, 14, or 28 days post injection. Control mice were injected with saline and killed 7 days later. Results are mean ± SEM of percent area stained in the CA3 region of the hippocampus, indicating significant reductions in diffuse Aβ from 3-14 days. X-axis label C = saline injected control mice killed 7 days post injection; * p < .05.
Gliosis, a possible link to the mechanism of Aβ reduction in APP mice, was analyzed by looking at reactive glial markers. Representative micrographs from the CA3 subregion of the hippocampus of APP mice are presented in Figure 16. Reactive microglia were immunohistochemically analyzed for CD45 expression in both APP and nontransgenic mice. Saline-injected APP mice demonstrated intense reactivity in conjunction with putative amyloid deposits (Fig. 16A). LPS treatment resulted in a widespread activation of microglia (Fig. 16B). LPS similarly activated microglia in nontransgenics (not shown). As shown in Panel C, the increase in CD45 immunostaining was time dependent ($F_{(9,48)} = 8.6, p < .0001$). Post hoc analysis indicated a statistically significant increase beginning at 3 days, peaking at 7 days, and remaining somewhat elevated throughout the time course to 28 days. Notably, the APP mice were significantly more reactive than their nontransgenic littermates at 7 days with CD45 immunostaining ($p < .01$).

Astrocytes in both APP and nontransgenic mice were detected using the astrocyte specific marker GFAP, as shown for the CA3 subregion in Figure 16. The hippocampus of saline-injected mice showed many GFAP+ astrocytes (APP mouse shown in Fig.16D). LPS treatment resulted in a widespread activation of astrocytes (Fig. 16E). The increase in GFAP immunostaining was time dependent ($F_{(9,48)} = 3.7, p < 0.005$), shown in Panel F. Post hoc analysis showed GFAP expression peaking at 7 days, then returning to control levels by 14 days. This is in contrast to CD45 which remained elevated through the 28 day time point. LPS injection into nontransgenic mice resulted in a mild increase in GFAP immunoreactivity compared to saline injection, which was not statistically significant.
FcγRII/III immunostaining of microglia was investigated following LPS injection. Figure 16G-H shows the CA3 subregion analysis of APP mice. Saline-injected mice showed no notable immunoreactivity. LPS treatment led to a widespread reaction in the injected hippocampus of APP mice (Fig. 16H). The increase in immunostaining was time dependent \( F_{(9,48)} = 1.8, p < .05 \), shown in Figure 16I. Post hoc analysis indicated significant elevation of FcγRII/III at 3 and 7 days post injection in APP mice. Levels returned to baseline by 14 days. Nontransgenic mice showed elevated reactivity as well, which was significant at 3 days when the data were analyzed separately from the transgenics.

*Figure 16. Time dependent glial reaction to LPS injection.* APP mice were injected with saline (A,D,G) or 4 µg of LPS (B,E,H). Micrographs are representative of a 7 day survival for both groups, imaging the CA3 region of the injected hippocampus. Immunostaining for CD45 (A,B) showed intense microglial reaction to LPS, which was significant from 3-28 days post injection (C). Immunostaining for GFAP (D,E) showed reactive astrocytes responding to LPS, which was significant at 7 days post injection (F). Immunostaining for FcγRII/III (G,H) showed upregulation of this receptor in response to LPS, which was significant from 3-7 days (I). Scale bar = 50 µm; x-axis label C = saline injected control mice; NT = nontransgenic; * \( p < .05 \) versus control for that genotype; ** \( p < .05 \) versus APP controls as well as compared to NT at the same time point.
Figure 16. Time dependent glial reaction to LPS injection.
The morphology of the microglia across time was examined with CD45 immunostaining (Fig. 17). Saline-injected APP mice showed predominantly lightly stained, resting, ramified microglia with thin delicate processes (Fig. 17A). Treatment with LPS resulted in a production of rounded, ameboid microglia, staining intensely with CD45, appearing 1-7 days post injection (Fig. 17B). Starting at 7 days and lasting though 28 days, microglia acquired a hyper-ramified, bushy appearance, with short thick processes stained heavily with CD45 (Fig. 17C). CD45-positive microglia, associated with putative amyloid deposits in saline-treated APP mice, have a bushy appearance and are heavily stained by CD45 antibodies (Fig. 17D). After LPS injection, ameboid microglia surrounded these putative plaques at 1, 3, and 7 days (Fig. 17E). The microglia resumed their bushy appearance at 7, 14, and 28 days, and remained widely distributed throughout the hippocampus as well as associated with putative amyloid plaques (Fig. 17F). Particularly at the 7 day time point, there was overlap with multiple morphologically different microglia being detected.
Figure 17. Altered microglial morphology in response to LPS. CD45 immunostaining was examined at each time point. Micrographs are from the CA1 molecular layer of APP mice injected with saline 7 days earlier (panels A,D), LPS 3 days earlier (panels B,E) or LPS 28 days earlier (panels C,F). Micrographs were chosen from a region free of putative amyloid plaques (A-C) or a field centered on a putative amyloid plaque (D-F). Scale bar = 50 µm.
Discussion

These findings provide evidence of an innate mechanism of diffuse Aβ removal present in APP transgenic mice. LPS injections into the hippocampus led to a time-dependent clearance of parenchymal Aβ. These results confirm those reported earlier by our lab and others (Dicarlo et al., 2001; Quinn et al., 2003). Congophilic plaque load was unaffected, suggesting these different pools of amyloid are selectively accessible by glial cells or other clearance mechanisms. Potential mechanisms for removal of diffuse Aβ include phagocytosis by glial cells, secretion of degradative enzymes, or facilitation of Aβ clearance from the brain by mechanisms other than phagocytosis. Scavenger receptors may represent the link between LPS and Aβ, as these receptors have been shown to bind both ligands, and can mediate Aβ phagocytosis in vitro (Husemann et al., 2002). Though both microglia and astroglia have phagocytic capabilities in vitro, the time course of Aβ reduction observed in these experiments most closely mirrors that of microglial activation. Furthermore, early time points were associated with a shift in microglial morphology to an ameboid shape that is associated with phagocytic capability in other systems.

Another marker for a phagocytic state of microglia is Fcγ receptor expression. FcγRII/III were up-regulated on microglial cells in response to LPS from 3-7 days post injection. Previous studies performed by our laboratory have demonstrated that anti-Aβ antibodies injected into the brain of APP mice stimulated microglia to remove Aβ in a process that appears at least partially Fc receptor mediated (Wilcock et al., 2003, 2004a,b). The endogenous ligand for Fcγ receptor is the Fc region of IgG. However, there is no evidence in the current studies that anti-LPS or anti-Aβ antibodies have been
produced in the LPS-injected mice, although such a possibility cannot be ruled out. On the other hand, it has been demonstrated that LPS can induce Fcγ expression in the absence of IgG, possibly as part of cell activation. In addition, Fc receptor expression by macrophages can be regulated by cytokines as well as by LPS (Amigorena et al., 1989; Lynch et al., 1990; Laszlo and Dickler, 1990; Loughlin et al., 1992; Keller et al., 1994). Thus, it is possible that the induction of FcγRII/III reflects another facet of microglial activation and not the specific presence of immunoglobulin.

The microglial response, though decreasing with time, remained significantly activated throughout the time course. At 28 days post injection, CD45+ microglia were still significantly activated, and had adopted a hyper-ramified, bushy appearance. The glia were both plaque-associated as well as widespread throughout the injected hippocampus. It was interesting that the appearance of microglia at 28 days was very similar to those associated with plaques in our control, saline treated APP animals. Our experiments also demonstrated exacerbated gliosis after 7 days in the LPS dosed APP transgenics compared to their nontransgenic littermates. Both CD45+ microglia and GFAP+ astrocytes were more intensely immunolabeled in the transgenics. These results suggest a role for Aβ in the inflammatory response.

An intriguing observation occurred at the 28 day time point. Aβ levels, which had been significantly reduced from 3-14 days post injection accumulated rapidly between 2 and 4 weeks after LPS administration, and returned to pre-injection levels after one month. The original Aβ burden in these mice developed over their lifespan of 16-17 months. This finding is highly significant because it suggests that amyloid pools are more labile than previously assumed. It is unclear whether this rapid re-accumulation
resulted from cessation of Aβ removal mechanisms, enhancement of Aβ deposition or both. If the LPS molecule is required for sufficient microglial activation and Aβ removal, complete clearance of LPS might signal cessation of removal mechanisms as the immune response is no longer stimulated. On the other hand, evidence of enhanced APP processing in response to insult is well documented. Chronic neuroinflammation and trauma-induced injury models both showed increases in APP and its processing (Siman et al., 1989; Kawarabayashi et al., 1991; Griffin et al., 1994; Banati et al., 1995; Hauss-Wegrzyniak et al., 1998; Sugaya et al., 1998). Kainate lesions also increased APP expression in astrocytes (Siman et al., 1989; Wright et al., 1999). Enhanced α-secretase as well as presenilin activity were shown in other injury models (Brugg et al., 1995; Pennypacker et al., 1999). Future experiments will examine expression and proteolytic processing of APP after LPS administration to ascertain whether they play a role in this rapid re-accumulation of AB burden.
Chapter 4

Dexamethasone Suppresses LPS-Induced Microglial Activation and Amyloid Clearance in APP Transgenic Mice

Abstract

Inflammation has been argued to play a primary role in the pathogenesis of Alzheimer’s disease (AD). Mice transgenic for mutant human amyloid precursor protein (APP) have amyloid deposits, gliosis, and cognitive impairment, without apparent neurodegeneration. Previous efforts by our group to use lipopolysaccharide (LPS) to elicit neurodegeneration in APP mice were unsuccessful. Instead we saw a surprising reduction in Aβ burden concurrent with the inflammatory response. In studies described here, we clarify the mechanisms involved in LPS mediated removal of Aβ, using dexamethasone to inhibit the microglia response. APP mice were intrahippocampally injected with LPS and survived 3 or 7 days with or without dexamethasone co-treatment. Brain tissue was then analyzed by immunohistochemistry and quantitative real time PCR. Total Aβ burden was reduced 7 days after LPS injection; this was prevented by co-treatment with dexamethasone. Markers of general microglial activation, CD45 and complement receptor 3, were increased by LPS and inhibited by dexamethasone. In contrast, the Fcγ receptors II/III and scavenger receptor A were increased by LPS but unaffected by dexamethasone treatment. The implications of the glial response to LPS and dexamethasone are two-fold. First, cells do not require general activation in order to
upregulate their expression of receptors capable of mediating phagocytosis. However, in order to effectively clear out foreign or toxic materials (such as LPS, Aβ, etc.) general cell activation is necessary.

Introduction

Alzheimer’s disease (AD) is one of many neurodegenerative disorders marked by chronic inflammation (Akiyama et al., 2000). Although amyloid plaques in human AD brain are surrounded by reactive astrocytes and microglia, the cellular response is not well understood (Itagaki et al., 1989). Acute inflammation can be neuroprotective, aiding in removal of pathogens and protecting cells (Streit, 2002). Conversely, excessive, chronic inflammation is thought to cause neuron death via an autotoxic mechanism (McGeer & McGeer, 2002). Current transgenic mouse models replicate the amyloid pathology, glial response, and cognitive impairment of AD, without apparent neurodegeneration (Gordon et al., 2001, 2002).

Based on the inflammation hypothesis of AD, we have created a model using LPS intracranial injections into mice carrying a mutant human amyloid precursor protein (APP) transgene. LPS is expressed on the cell wall of Gram-negative bacteria, and can cause an inflammatory response by the host (Palsson-McDermott & O’Neil, 2004). Once in the host, LPS binds to circulating LPS binding protein, which subsequently transfers the LPS to cell membrane bound cluster differentiation marker CD14 (Heumann & Roger, 2002). Toll like receptor 4 (TLR4), as well as myeloid differentiation protein MD2, form a complex through which LPS:CD14 can transduce a signal (Thomas et al., 2002).
We have reported previously that direct injection of lipopolysaccharide (LPS) into the hippocampus of APP mice resulted in reductions in Aβ burden (DiCarlo et al., 2001; Herber et al., 2004b). Similar findings were seen after intraperitoneal injections of LPS (Quinn et al., 2003) although systemic administration to young mice may have the opposite effect and precipitate amyloid deposits where none previously existed (Qiao et al., 2001). A potential mechanism of Aβ removal includes phagocytosis (D'Andrea et al., 2004). In vitro studies showed that both microglia and astrocytes respond to LPS with phagocytosis, as well as with production of potentially cytotoxic agents such as cytokines and reactive oxygen species (Kalmar et al., 2001; Shaffer et al., 1995). Wyss-Coray and colleagues also showed reductions in amyloid deposits when brain slices from APP mice were coated with astrocytes (2003).

Here we describe experiments that explore the glial contribution to the LPS associated Aβ reductions. Intrahippocampal LPS injections reduced Aβ burden and stimulated microgliosis. The microglia response was then inhibited with dexamethasone, resulting in Aβ burdens at control levels.

Materials and Methods

Mouse Strains

Transgenic mice were bred to develop Alzheimer’s like pathology using Tg2576 APP mice as described previously (Holcomb et al., 1998). APP mice and their nontransgenic littermates aged 17 months were used in this study. Animals were group-housed prior to surgery under a 12 hr light-dark cycle with free access to chow and water.
Intrahippocampal Injections

Mice were anesthetized using isoflurane and immobilized in a stereotaxic apparatus. A single, one microliter injection of either saline or 4 µg/µl of LPS (Salmonella abortus equi, Sigma, St. Louis, MO) was delivered over a two minute period into the hippocampus (coordinates from bregma: -2.7 mm posterior, +/-2.5 mm lateral, and -3.0 mm ventral). The incision was closed with wound clips, isoflurane was discontinued, and the animal was revived under ambient conditions. All mice completely recovered within five minutes. Animals were singly housed for the post treatment survival period under standard vivarium conditions. We used at least 5 mice (5-10) for each genotype and treatment, balanced for gender. Post LPS injection, the survival period was either 3 or 7 days.

Drug Administration

Two different time points were evaluated. A 3 day study was conducted after intrahippocampal LPS injection. Within one hour of injection, mice were administered dexamethasone (5 mg/kg, ip), followed by twice daily administration for a total of 3 days. A 7 day study was conducted by pre-treating with dexamethasone for 24 hours, injecting with LPS, then continuing twice daily dexamethasone for 7 days.

Tissue Preparation

Mice were overdosed with pentobarbital, and then perfused transcardially with 25 ml of normal saline. Tissue was collected for RNA analysis by removing the hippocampus and storing at -80°C. For immunohistochemistry, the brain was post fixed in 4% paraformaldehyde solution for 24 hours, then processed through a cryoprotection schedule of 10, 20, and 30% sucrose. Brains were sectioned horizontally at 25 µm on a
freezing stage using a sliding microtome. Sections were then stored in Dulbecco’s phosphate buffered saline pH 7.4 (DPBS) with 100 mM sodium azide at 4°C.

**Histology**

Immunohistochemical analysis was performed for each marker using six, 25 µm free-floating sections spaced 200 µm apart through the hippocampus. Details of this procedure are described elsewhere (Gordon et al., 1997). Briefly, sections were blocked for endogenous peroxidases (10% methanol and 3% hydrogen peroxide in 80% DPBS), washed with DPBS, and then permeabilized (100 mM lysine, 0.2% triton x-100, 4% normal serum in DPBS). Sections were then incubated overnight in the appropriate primary antibody (Table 6). The following day, sections were washed, and then incubated in appropriate biotinylated secondary antibody. After another cycle of washes, the tissue was incubated with Vectastain® Elite® ABC kit (Vector Laboratories, Burlingame, CA). The tissue was then washed and stained with a nickel: diaminobenzidine: peroxide system, followed by final washes. In the case of Aβ immunostaining, nickel enhancement of the color development was not used. The extent of nonspecific binding was assessed in the absence of primary antibodies for all assays. Sections were mounted onto slides, dehydrated, and cover-slipped with DPX (E.M. Sciences, Fort Washington, PA).

Slide-mounted untreated sections were stained with Congo red to detect compact amyloid plaques. Slides were briefly hydrated in water, and then incubated in alkaline alcoholic saturated sodium chloride (AASSC) for 20 minutes, followed by 30 minutes in 0.2% Congo red in AASSC. Slides were then quickly dehydrated through a graded ethanol series, cleared in xylene, and cover-slipped.
**Table 6. Primary Antibodies Used for Immunohistochemistry**

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Immunostained sections were imaged at 100x magnification, focusing on the CA1, CA3, dentate gyrus, and hippocampal fissure subregions of the hippocampus, as well as the anterior cortex. Images were then analyzed for area percent positive stain using Image-Pro® Plus software (MediaCybernetics, Silver Spring, MD). Area percent stained was calculated for each individual region of the hippocampus, and averaged to calculate the staining for the entire hippocampus. Results were averaged for all sections from each animal, and the treatment groups compared by one way analysis of variance (ANOVA) followed by post hoc analysis using Fisher’s protected least squares difference (PLSD, significance at $p < .05$). In some cases, results are reported in terms of fold change from control levels. In other instances, the ratio of staining in the hippocampus to that found in the anterior cortex was used to normalize for the variable amyloid deposition in individual mice.
**RNA Analysis**

Tissue from the hippocampus was analyzed for mRNA expression by reverse transcription followed by quantitative real time polymerase chain reaction (qRT-PCR), as previously described (Dickey et al., 2003). RNA was extracted from the injected hippocampus using Qiagen’s Rneasy® procedure (Valencia, CA). The purified RNA was then assayed using Molecular Probes RiboGreen® RNA quantitation kit (Molecular Probes, Eugene, OR). Each sample was then diluted in water to a final concentration of 50 ng/µl or 5 ng/µl. The 50 ng/µl samples were run on a 1% agarose gel using ethidium bromide/ultraviolet detection to verify RNA integrity.

A composite of all RNA samples was made in order to prepare a standard curve ranging from 0.2 – 50 ng/µl. Reverse transcription with mMLV (Invitrogen, Carlsbad, CA) was then performed on the standard dilutions and 5 ng of each sample RNA solution. The resulting cDNA was then subjected to qRT-PCR using SYBR® Green PCR Master Mix (Molecular Probes, Eugene, OR). Table 7 contains the primer sequences used in the qRT-PCR reaction. For each mRNA of interest, a melt curve analysis of the qRT-PCR product was performed after cycling was complete. Fluorescence was read in 1°C increments from 56-99°C. Primer pairs were designed to yield a single, specific product, with no evidence of primer-dimer formation.
For each mRNA of interest, the threshold cycle \([C(T)]\) values from the qRT-PCR were determined. To create a linear curve, the log of the ng of RNA in each standard was plotted against the average \(C(T)\) value (each sample and standard was analyzed in triplicate). Next, the log of the ng of RNA in each sample were calculated, and then converted to ng of RNA. For a given reverse transcription, all targets of interest were normalized to results for 18S ribosomal RNA (18S rRNA) for the same sample to control for variations in the starting RNA concentration and reaction efficiency. The ratio of the ng of target RNA to ng of 18S rRNA was calculated, and the results subjected to one way ANOVA, followed by Fisher’s PLSD with significance taken at \(p < .05\). The final results are presented as fold change versus saline injected control mice.
Results

Intrahippocampal injections of LPS were made into APP transgenic mice and the subsequent neuroinflammatory effects on amyloid load evaluated. We first looked at brain amyloid burden, considering both total Aβ and Congophilic amyloid deposits. Figure 18A-D shows the changes in total Aβ burden after treatment. The micrographs are representative of the injected hippocampus of mice which survived 7 days. Control mice showed substantial amounts of Aβ immunostaining, including focal aggregations scattered throughout the molecular layer of the CA subfields as well as laminar accumulations along the hippocampal fissure and outer molecular layer of the dentate gyrus (Fig. 18A). In contrast, LPS treated mice showed less Aβ staining throughout the hippocampus (Fig. 18B). Reductions in the laminar accumulations were particularly striking. Mice both injected with LPS and co-treated with dexamethasone showed many deposits and laminar accumulation (Fig. 18C), similar to control mice. The area percent positive stain was calculated for each region of the hippocampus, with the greatest effects seen at the hippocampal fissure. The average area percent of the entire hippocampus was compared to the amount of Aβ staining in the ipsilateral anterior cortex to control for inter-animal variability. The mean area percent ± standard error of the mean for the anterior cortex was not significantly different among the groups: 18.5 ± 3.6 saline, 18.8 ± 2.9 LPS, 14.8 ± 1.3 LPS-dexamethasone at 3 days; 12.5 ± 2.9 saline, 19.4 ± 2.2 LPS, 15.4 ± 2.2 LPS-dexamethasone at 7 days. Statistical analysis revealed that the LPS treated group surviving 7 days had significant reductions in Aβ load in the hippocampus compared to saline injected control mice. This effect was completely blocked by
concurrent administration of dexamethasone. No changes in Aβ load were found at 3
days in any of the groups (Fig. 18D).

The effect of LPS injection on compact plaques was evaluated using Congo red
staining, shown in Figure 18E-H. The micrographs are representative of the injected
hippocampus of mice which survived 7 days. Saline injected mice showed several focal
deposits along the hippocampal fissure (Fig. 18E). LPS treated mice showed similar
numbers and sizes of deposits in the hippocampus in the absence (Fig. 18F) and presence
(Fig. 18G) of dexamethasone at both 3 and 7 days. These results were analyzed in the
same manner as for Aβ immunostaining and are presented in Figure 18H. There were no
statistically significant effects of LPS or dexamethasone on Congo red levels at either
time point.
Figure 18. LPS injection reduced diffuse but not compact amyloid deposits and was reversed by dexamethasone. APP mice were injected with saline, LPS, or LPS plus concurrent dexamethasone treatment for 3 or 7 days. Micrographs are representative of animals survived 7 days. Immunostaining for Aβ in saline injected mice showed many deposits throughout the hippocampus (A). LPS injection reduced the total amount of Aβ staining in the hippocampus (B). LPS injection plus dexamethasone co-treatment resulted in many Aβ deposits (C), similar to saline injected mice. The LPS-induced reduction in Aβ was significant at the 7 day time point and blocked by dexamethasone (D). In contrast, similar amounts of Congophilic deposits were seen in saline injected (E), LPS injected (F), and LPS plus dexamethasone co-treated groups (G). There was no significant difference in Congo red staining between any of the treatment groups. Scale bar = 500 μm, * p < .05.
Figure 18. LPS injection reduced diffuse but not compact amyloid deposits and was reversed by dexamethasone.
Two markers of general microglial activation, CD45 and CR3 were analyzed by immunohistochemistry and are shown in Figure 19. All micrographs are from the injected hippocampus of mice which survived 7 days. CD45 (Fig. 19A) and CR3 (Fig. 19E) stained reactive microglia in association with putative amyloid deposits in saline injected mice. LPS injection resulted in a widespread activation of both CD45-positive (Fig. 19B) and CR3-positive (Fig. 19F) microglia, throughout the hippocampus and nearby cortices. The insets in these panels show higher power micrographs of the microglial morphology. Dexamethasone treatment of LPS-injected mice blocked the elevation of CD45 (Fig. 19C) and CR3 (Fig. 19G) staining, similar to that observed in saline injected mice. Quantitation of the fold change of the area percent stained in the dentate gyrus compared to saline injected control mice is shown in Figure 19D for CD45 and Figure 19H for CR3. Similar effects were seen on all regions of the hippocampus, but the injection site was typically in the hilus of the dentate gyrus. Two way ANOVA for CD45 revealed significant effects of treatment ($F_{2,46} = 39, p < .0001$), time ($F_{1,46} = 5.8, p = .02$), and interaction ($F_{2,46} = 14, p < .0001$). Similar results for CR3 showed significant effects of treatment ($F_{2,46} = 17, p < .0001$), time ($F_{1,46} = 76, p < .0001$), and interaction ($F_{2,46} = 3.5, p = .04$). Post hoc analysis confirmed that both the CD45 and CR3 responses were significantly elevated at 3 days and peaked at 7 days after LPS injection. Dexamethasone inhibited this reaction at both time points; levels were no different from controls.
Figure 19. CD45 and CR3 are induced by LPS and inhibited by co-treatment with dexamethasone. APP mice were injected with saline, LPS, or LPS with concurrent dexamethasone treatment for 3 or 7 days. Micrographs are representative of animals survived 7 days. Saline injected animals showed detectable levels of CD45 (A) and CR3 (E). Both CD45 (B) and CR3 (F) showed widespread increases after LPS injection; the insets depict higher magnification of reactive microglia. CD45 and CR3 also showed an effect of time; both were significantly elevated at 7 days compared to 3 days after LPS injection. Dexamethasone co-treatment inhibited CD45 (C) and CR3 (G) at both time points. Quantitation of the fold change versus saline injected controls is presented in Panel D (CD45) and Panel H (CR3). Scale bar = 500 µm, * $p < .05$ compared to saline injected controls, # $p < .05$ compared to the 3 day time point.
Figure 19. CD45 and CR3 are induced by LPS and inhibited by co-treatment with dexamethasone.
Two markers of the phagocytic capabilities of microglia, the low affinity Fc gamma receptors FcγRII/III and scavenger receptor SRA, were also examined by immunohistochemistry and are shown in Figure 20. All micrographs are from the injected hippocampus of mice which survived 7 days. FcγRII/III staining was associated with putative amyloid deposits, particularly along the hippocampal fissure in saline injected mice (Fig. 20A). LPS injection resulted in a marked response throughout the hippocampus (Fig. 20B). Dexamethasone treatment of LPS injected mice had no effect, and many FcγRII/III-positive microglia were seen (Fig. 20C), similar to mice who had received LPS injection only. The insets in Figure 20C and G show higher power micrographs of the microglia. Quantitation of the fold change of the area percent stained in the dentate gyrus compared to saline injected control mice is reported. FcγRII/III staining in LPS injected and LPS-dexamethasone co-treated mice after 3 and 7 days were significantly elevated compared to controls when analyzed by ANOVA (Fig. 20D: $F_{(2,46)} = 7.8, p = .001$). There was no significant effect of time, with similar increases at both 3 and 7 days. SRA immunostaining results were similar to FcγRII/III. Saline injected mice showed little SRA immunoreactivity (Fig. 20E). LPS injection resulted in a widespread reaction throughout the hippocampus (Fig. 20F). Dexamethasone treatment of LPS injected mice had no effect, and many SRA-positive microglia were seen (Fig. 20G), similar to mice who had received LPS injection only. Statistical analysis of SRA staining using ANOVA revealed a significant effect of LPS treatment (Fig. 20H: $F_{(2,46)} = 4.0, p = .03$). Note that because immunostaining in the control condition was very low, fold inductions for this marker are extremely large, and highly variable. SRA levels in LPS- injected and LPS plus dexamethasone co-treated mice were increased by 200 fold.
after 3 days, and 100 fold after 7 days. An insignificant effect of time was noted, with similar inductions of SRA at 3 and 7 days.

*Figure 20. FcγRII/III and SRA are induced by LPS but not inhibited by co-treatment with dexamethasone.* APP mice were injected with saline, LPS, or LPS with concurrent dexamethasone treatment for 3 or 7 days. Micrographs are representative of animals survived 7 days. Low levels of FcγRII/III were seen in saline injected mice (A). No SRA staining is evident after saline injection (E). Both FcγRII/III (B) and SRA (F) showed widespread increases after LPS injection. Only a mild, insignificant effect of time was noted. Dexamethasone co-treatment did not inhibit FcγRII/III (C) or SRA (G) levels at either time point; the insets depict higher magnification of microglia. Quantitation of the fold change versus saline injected controls is presented in Panel D (FcγRII/III) and Panel H (SRA). Scale bar = 500 µm, * p < .05 compared to saline injected controls.
Figure 20. FcγRII/III and SRA are induced by LPS but not inhibited by co-treatment with dexamethasone.
Based on previous findings that most RNAs peaked at 3 days (Herber et al., 2004a), several markers were examined at that time point, as shown in Figure 21. The relative expression of CD45 after LPS injection or LPS-dexamethasone co-treatment (compared to saline injected control mice) is shown in Figure 21A. ANOVA revealed a significant effect of dose ($F_{2,17} = 4.5$, $p = .03$). Post hoc analysis showed LPS significantly increased CD45 mRNA levels, which was inhibited by dexamethasone co-treatment. Results from post hoc analysis of the cytokine interleukin 1 beta (IL1β) levels also showed a significant increase due to LPS, which was ameliorated by dexamethasone (Fig. 21B). In contrast, FcγRIIb (Fig. 21C) and SRA (Fig. 21D) mRNA were induced by LPS and unaffected by dexamethasone co-treatment. ANOVA revealed a significant effect of treatment for both markers (FcγRIIb: $F_{2,17} = 4.8$, $p = .02$; SRA: $F_{2,17} = 8.4$, $p = .003$). Post hoc analysis showed LPS and LPS plus dexamethasone co-treatment groups were both significantly elevated compared to controls for FcγRIIb and SRA.

Figure 21. LPS stimulated gene transcripts have a pattern similar to protein expression. APP mice were injected with saline, LPS, or LPS with concurrent dexamethasone treatment for 3 days. Graphs depict RNA analysis of the injected hippocampus compared to saline injected controls. CD45 (A) and IL1β (B) transcripts were induced by LPS and inhibited by dexamethasone. In contrast, FcγRIIb (C) and SRA (D) transcripts were induced by LPS but not inhibited by concurrent dexamethasone treatment. * $p < .05$. 

103
Figure 21. LPS stimulated gene transcripts have a pattern similar to protein expression.
Discussion

Here we have shown the ability of the innate immune system to remove Aβ from the brains of APP transgenic mice. Intrahippocampal injections of LPS reduced the total Aβ burden without affecting Congophilic plaques, confirming previous reports (DiCarlo et al., 2001; Herber et al., 2004b). These findings indicate that the diffuse Aβ material is more sensitive to whatever mechanisms are activated by the LPS injection compared to compacted material. As a first attempt to identify such mechanisms, we partially inhibited the inflammatory reaction to LPS injection with systemic dexamethasone administration. This resulted in attenuation of the amyloid clearance by LPS, and a significant reduction in most microglial markers which were elevated by LPS. Therefore it is likely that the glial reaction to LPS was responsible for the removal of diffuse Aβ.

In an effort to characterize the glial response to LPS, we evaluated both general markers of activation (CD45 and CR3) as well as receptors involved in phagocytosis (FcγRII/III and SRA). Though CD45, CR3, FcγRII/III, and SRA were all induced by LPS injection, only CD45 and CR3 were inhibited by subsequent dexamethasone treatment. RNA analysis from the hippocampus of LPS injected versus LPS plus dexamethasone co-treated mice confirmed the immunohistochemistry results. The inhibition of CD45 by dexamethasone has also been reported after anti-Aβ antibody treatment, thereby preventing antibody-mediated removal of Aβ (Wilcock et al., 2004a). Similarly, in another report, dexamethasone inhibited CR3 levels after LPS injection into the substantia nigra (Castano et al., 2002). In contrast, glucocorticoids actually enhanced certain Fc receptor subtypes in some models (Kizaki et al., 1996; Sivo et al., 1993;
Yamaguchi et al., 2001). We have previously shown that FcγRII/III and SRA expression after LPS injection was transient, peaking from 3-7 days and returning to baseline by 14 days post injection (Herber et al., 2004a,b). This was in stark contrast to CD45 and CR3 which remained elevated out to 28 days post injection. Promoter analysis of all four genes shows little similarity which may account for their differential regulation in response to LPS and glucocorticoid treatment (Grewal et al., 2001; Nishimura et al., 2001; Timon & Beverley, 2001).

Both microglia and astrocytes are capable of phagocytosing Aβ *in vitro* and in slice preparations (Shaffer et al., 1995; Wyss-Coray et al., 2003). The mechanism by which this is accomplished *in vivo* is unclear and as yet unproven. Scavenger receptors, β-integrins, complement proteins, lipoprotein receptor related proteins, and receptors of advanced glycation end products can all bind Aβ and mediate endocytosis (Herz & Strickland, 2001; Huseman et al., 2002; Koehigsknecht & Landreth, 2004; Schmidt et al., 2001). Although FcγRII/III are increased in our LPS model, we have no direct evidence of antibody production, and upregulation of these receptors was probably due to the general activation of the cell in preparation for initiation of an acquired immune response (Amigorena et al., 1989; Iwasaki & Medzhitov, 2004; Keller et al., 1994; Laszlo & Dickler, 1990). Though scavenger receptor B (SRB) has been implicated in AD (Coraci et al., 2002), no information is available for SRA. We previously examined SRB mRNA following LPS injection and saw only a small increase at 6 hr post injection, and therefore have shifted focus to SRA (Herber et al., 2004a). SRA seems to be at least partially responsible for astrocyte- mediate amyloid removal, though similar results with microglia are not available (Wyss-Coray et al., 2003).
The implications of the glial response to LPS and dexamethasone are two-fold. First, cells do not require general activation in order to upregulate their expression of receptors capable of mediating phagocytosis. However, in order to effectively clear out foreign or toxic materials (such as LPS, Aβ, etc.) general cell activation is necessary.
Conclusions

Alzheimer’s disease was first described by Alois Alzheimer in 1907 in an article entitled, “On an Unusual Illness of the Cerebral Cortex.” In this seminal work, Alzheimer describes a 51 year old female patient who suffered from severe dementia. Upon autopsy, her brain was examined and found to be grossly atrophied. Histopathology revealed,

“the nucleus and the cell itself disintegrate and only a tangle of fibrils indicates the place where a neuron was previously located… Many neurons, especially in the upper layer, have completely disappeared. Distributed all over the cortex, but especially numerous in the upper layers, there are minute military foci which are caused by the deposition of a special substance in the cortex.”

The terms neurofibrillary tangles (NFT) and plaques, or senile plaques, have since been coined for the typical pathology of AD. Additional aspects of the disease include inflammation and alterations in the cerebrovasculature (Akiyama et al., 2000; Rhodin & Thomas, 2001; de la Torre, 2002). Several hypotheses have been proposed over the past century as to the cause of the disease. Recent therapeutic developments aim to remove or prevent amyloid deposition, based on the amyloid hypothesis of AD. This hypothesis proposes “amyloid deposition as the central event in the aetiology of Alzheimer’s disease” (Hardy & Allsop, 1991). Such a view presumes neurofibrillary tangles, neurodegeneration, and inflammation (the other pathological components of AD) to be a consequence of amyloid deposition.
The major component of plaques is the Aβ peptide, which can aggregate to form amyloid deposits. This peptide originates from the amyloid precursor protein (APP), as a result of proteolytic cleavage by beta- and gamma-secretases (Nunan & Small, 2002). The Aβ peptide varies in length from 37-43 amino acids. Aβ1-40 is the predominant species, but Aβ1-42 is more hydrophobic and therefore more readily forms fibrils. In support of the amyloid hypothesis are the discoveries of several genetic mutations, all of which cause enhanced deposition of amyloid and ultimately lead to autosomal dominant AD, particularly at a young age (pre-senile). These mutations make up the portion of the population with familial Alzheimer’s disease, and account for approximately 5-10% of all AD cases. In 1991 Goate and colleagues reported mutations located on human chromosome 21 which ultimately mapped to the APP gene. A single amino acid substitution of valine to isoleucine, close to the carboxy terminus of the Aβ peptide, results in a more hydrophobic, and possibly more fibrillogenic, species with the added potential for enhanced gamma secretase cleavage. Over the past 15 years, many mutations have been described near the c-terminus of Aβ, such as the Austrian, French, Florida, London, and Australian varieties (Nunan & Small, 2002). Mutations located in the middle of the Aβ peptide cause cerebral amyloid angiopathy (Van Nostrand et al., 2002). A double substitution at the N-terminus of Aβ (K670N, M671L), known as the Swedish mutation, leads to enhanced cleavage by beta secretase, and is the genetic mutation used in the Tg2576 transgenic mice described in our studies (Hsiao et al., 1996). These mice show age-associated amyloid deposition, reactive gliosis, and cognitive impairments, much like AD. Trisomy 21 leads to a triple dose of APP, and AD pathology can be seen in older Down’s patients, even though the gene is not mutated.
In 1995, mutations in two additional genes were identified as causal for AD. Sherrington and colleagues found missense mutations in the gene encoding presenilin 1 (PS1), located on chromosome 14, which led to autosomal dominant AD. That same year Levy-Lehad and colleagues reported mutations in a similar gene, presenilin 2 (PS2), located on chromosome 1, which also led to autosomal dominant AD. Greater than 85 mutations in the presenilins have been identified in familial AD. Presenilin is part of the gamma-secretase complex, which proteolytically cleaves APP in conjunction with beta-secretase to form Aβ (De Strooper, 2003). Transgenic mice carrying a PS1 mutation (M146L, line 5.1) show little pathology, with no amyloid deposition (Duff et al., 1996). However, breeding the Tg2576 APP mice with PS1 mice leads to accelerated amyloid deposition and cognitive dysfunction in mice bearing both mutations compared to mice with mutations in APP only (Holcomb et al., 1998; Gordon et al., 2001; 2002).

Associated with increased risk for AD are allelic forms of apolipoprotein E (APOE; Mahley & Huang, 2004). There are three APOE alleles expressed in humans (APOE2, 3, and 4) which are involved in lipid and cholesterol transport and homeostasis. The predominant allele is APOE3. Corder and colleagues identified a gene-dosing effect of the APOE alleles in 1993. An increased risk of late onset (>65 years old) Alzheimer’s disease was associated with the E4 allele. In cases of AD with APOE4, there is increased plaque and tangle density as well as an earlier age of onset compared with the E3 allele (Marz et al., 1996). Although the mechanism remains uncertain, Colton and colleagues have demonstrated a link between the APOE4 allele and exaggerated innate immune activation (Colton et al., 2002a,b, 2004; Czapiga & Colton, 2003).
The genetic mutations associated with familial AD as well as the gene dosing effects of APOE4 all increase the amyloid burden of the brain and are causal or associated with increased risk of AD. Though there are identifiable mutations in tau which cause tangle formation and lead to frontotemporal dementia or Pick’s disease, none of them are associated with AD (Friedhoff et al., 2000). Thus, the genetic linkage to amyloid deposition has further strengthened the amyloid hypothesis of AD.

Another approach to the study of AD is the inflammation hypothesis (McGeer & McGeer, 1995). According to this line of reasoning, it is not the lesions in AD brain (plaques, tangles, and neuron loss) but rather the inflammatory response to such lesions that leads to neuron loss and the clinical manifestations of the disease. Originally described by Celsus in the first century A.D., peripheral inflammation is characterized by rubor (redness), calor (heat), dolor (pain), and tumor (swelling). These responses are secondary to the primary reaction of the tissue. The primary process is characterized by both acute and chronic phases (Hardman et al., 2001). During the acute phase, tissue damage causes the release of cellular contents which triggers changes in the local vasculature as well as migration of leukocytes and macrophages to the site. The infiltrating immune cells can then release chemical mediators such as histamine, prostaglandins, leukotrienes, and cytokines. These chemical and cellular mediators of inflammation fight off infection as well as destroy damaged and infected cells. There are three outcomes to injury: the host wins and inflammation is resolved, the host loses and dies, or there is a stalemate where chronic inflammation persists. This chronic phase is characterized by macrophage activation and release of chemical mediators which can
ultimately cause tissue damage. Typical examples of peripheral chronic inflammation are arthritis and inflammatory bowel disease.

In the brain, swelling is the only secondary inflammatory response seen (no redness, pain, or heat). Swelling is seen only under extreme circumstances such as after traumatic brain injury or during encephalitis. However swelling is not seen in neurodegenerative conditions such as Alzheimer’s or Parkinson’s diseases, or multiple sclerosis. Instead, a more subtle inflammatory process can predominate in the brain, as characterized by reactive glia and the products they produce. A classic example of this type of silent brain inflammation is Alzheimer’s disease. This process is mainly mediated by the innate immune system, with the endogenous microglia as the tissue specific macrophages. B and T cells are largely absent from the brain and thus adaptive immune activation does not play a major role in the inflammation of AD (Akiyama et al., 2000). Similar to peripheral inflammation, there are acute and chronic inflammatory responses in the brain, resulting in microglial activation, release of chemical mediators, and the potential for neuron death. Cell death in the CNS is problematic due to the fact that neurons do not divide/replicate, and there is little repopulation by stem cells. If a neuron dies as a result of an inflammatory process, the lesion is permanent.

The exact mechanism of neuron death due to inflammation is probably a combination of factors. Reactive microglia, like all macrophages can produce a respiratory burst as well as cytotoxic elements such as complement proteins, cytokines, and prostaglandins (Colton et al., 1992; 2000; Colton & Gilbert, 1993; Colton & Chernyshev, 1996; Czapiga & Colton, 1996; Akiyama et al., 2000). In vitro, release of this chemical milieu can be triggered by phagocytic activities and/or exposure to
complement proteins, cytokines, prostaglandins, and microbial products (such as LPS), as well as the Aβ peptide (McGeer & McGeer, 1995). The respiratory burst (leading to release of oxygen free radicals and nitric oxide) causes oxidative damage to proteins and the subsequent stress leads to apoptotic cascades and neuron death. The complement cascade, both classical and alternative, can ultimately lead to formation of the membrane attack complex which participates in cell lysis (Shen & Meri, 2003). Cytokines and prostaglandins reinforce the reaction of microglia in a self perpetuating cascade as the inflammation remains unresolved. The accumulation of amyloid deposits suggests that microglia are unable to remove the deposits. Microglia are associated with amyloid plaques and may be experiencing “frustrated phagocytosis” as they are unable to effective clear the material (Colton et al., 2000; Fonsesca et al., 2004; Henson, 1971). The microglia therefore remain activated in a chronic inflammatory response. Though direct toxicity to neurons due to amyloid deposits and tangle formation is probable (Canevari et al., 2004; Davies, 2000), the progression may be slow without the additional inflammatory stimulus to intensify the process (McGeer & McGeer, 1995). Bystander lysis may also occur whereby undamaged neurons are killed as a part of this uncontrolled/chronic inflammatory response. Though reactive microglia have never been shown to kill healthy neurons in vivo, a chronic inflammatory cascade may be neurotoxic in AD. McGeer and colleagues (1995) propose an autotoxic loop which could lead to neuron death, shown in Figure 22.
Figure 22. Autotoxic mechanisms in Alzheimer’s Disease.

Supporting the inflammation hypothesis are studies showing multiple inflammatory cascades active in the AD brain including complement, cytokines and chemokines, prostanoids, acute phase proteins, and free radicals (Akiyama et al., 2000). Plaques and tangles are both inert, insoluble aggregates \textit{in vivo} and glia mount an inflammatory response to these aggregates. Studies in the 1980s of human post mortem AD brain showed reactive microglia and infiltrating T-cells (McGeer et al., 1987; 1988 a,b; Itagaki et al., 1988). Subsequently, the brain inflammatory response was correlated to AD and cognitive decline. Lue et al. (1996) measured plaque and tangle load as well as inflammatory markers such as complement proteins and MHCII in three groups: normal controls, high pathology controls (HPC), and overt AD patients. Both the HPC and AD brains had pathological diagnosis of AD with significant amounts of plaques and tangles. However, the HPC group had no synaptic loss, no overall brain atrophy, and significantly less inflammatory reaction. These findings led the authors to conclude:
“…of all the pathological variables examined, the best predictors of synaptic changes (i.e. the variables that accounted for the highest proportions of synapse variance) are those related to inflammation, C5b9 immunoreactivity, and activated LN3+ microglia. Taken together, these data suggest that elderly patients may present at autopsy with profuse cortical plaques and entorhinal cortex NFTs but may not evidence synaptic loss unless these changes are accompanied by inflammatory reactions. Inflammation may therefore be one of the final common pathways through which Aβ deposits and NFTs manifest their neurodegenerative effects…”

A similar study in 2003 by Vehmas et al. demonstrated a change in gliosis associated with the transition from probable to definite AD. Microgliosis was elevated in both groups compared to normal controls, with higher levels in the definite AD group. Astrogliosis was an even better correlate of the disease, with significantly elevated GFAP levels in definite AD compared to possible AD. Inflammatory gliosis has proven to be a robust finding in AD brain, and is also seen in amyloid depositing transgenic mice (Gordon et al., 2002).

In support of the inflammation hypothesis are several epidemiological studies showing a decreased risk for AD with anti-inflammatory drug use (reviewed in McGeer et al., 1996). Early studies examined arthritis as a factor in AD and established an overall 0.556 (osteoarthritis) and 0.194 (rheumatoid arthritis) odds ratio (OR) indicating decreased risk for AD and possible protective effects of the anti-inflammatory drugs commonly used by people with arthritis. When drug use was considered as a factor in AD, both steroids (OR = 0.656) and NSAIDS (OR = 0.496) decreased AD risk. A recent study conducted in the Netherlands considered exact drug and therapy duration as factors in AD (In ‘t Veld et al., 1998). The odds ratio overall was 1.0. However, when the data was stratified for age, a protective effect was seen in persons less than 85 years old (OR =
0.53). Additionally, a greater protective effect was seen in patients with more than 6 months NSAID use. Thus, it seems that inhibiting inflammation could be beneficial in Alzheimer’s disease.

Based on these findings, NSAIDs have been administered to amyloid depositing transgenic mice, with impressive results. Ibuprofen and NCX-2216 (a nitric oxide donating flurbiprofen derivative) significantly reduced amyloid burden in doubly transgenic APP:PS1 mice (Jantzen et al., 2002). Similar results for ibuprofen have been reported (Lim et al., 2000; Yan et al., 2003), and Eriksen et al. (2003) demonstrated that multiple NSAIDs reduced soluble Aβ in singly transgenic APP mice. The actual Aβ lowering mechanism of NSAIDs is a matter of controversy (reviewed in Gasparini et al., 2004). In vitro, NSAIDS can inhibit Aβ aggregation, and decrease Aβ production. However, the Aβ lowering effects seem to be independent of cyclooxygenase (COX) activity as doses required to alter Aβ levels are much high than those necessary to inactivate the COX enzyme (Weggen et al., 2001). The Aβ lowering effects fo NSAIDs also seem to be independent of PPARγ activity. Ciglitazone, a PPARγ agonist, did not alter Aβ levels in vitro (Sagi et al., 2003), nor did pioglitazone affect amyloid deposition in vivo (unpublished data by Paul Jantzen in our lab).

Several NSAIDs have been used in human trials to establish effects on AD, though results to date have been discouraging. Rofecoxib, naproxyn, and a combination of diclofenac/misoprostol have all been eliminated as potential AD therapies in clinical trials (Aisen et al., 2003; Reines et al., 2004; Scharf et al., 1999). Currently under clinical investigation are ibuprofen, indomethacin, and celecoxib, listed on the US government’s web site of active clinical drug trials (http://clinicaltrials.gov).
However, not all inflammation is regarded as detrimental. As mentioned previously there are three potential outcomes to injury/insult: the host wins and inflammation is resolved, the host loses and dies, or a stalemate is reached and chronic inflammation ensues. The first outcome (the host wins, tissue is repaired, and inflammation is resolved) reveals the protective side of inflammation. In the brain, reactive glia can aid in removal of pathogens and cellular debris, as well as supply trophic factors. In AD, a key therapeutic strategy uses vaccination against the Aβ peptide and stimulates the immune system resulting in decreased amyloid burden (reviewed later in this discussion). Therefore, stimulating the immune system, including microglia, represents a potential means of removing brain amyloid in AD.

The model we used for the studies described in this dissertation combined both the amyloid and inflammation hypotheses of AD in an effort to create a system that more closely resembled the human condition. We injected the inflammatory agent lipopolysaccharide (LPS) into the hippocampus of various transgenic mice, in particular amyloid -depositing APP mice. LPS triggers the innate immune response leading to inflammation. The expected outcome was enhanced amyloid burden and/or neuron death, similar to that seen in human AD brain.

In the first study, we examined α7 nAChRs in amyloid- depositing mice and during neuroinflammatory conditions. Our desire to evaluate nicotinic receptors was based on several observations. First, cholinergic signaling is impaired in AD with declines in α7 nAChRs among other populations (Perry et al., 2001). Second, there have been numerous reports of nicotine’s protective effect for AD (reviewed in Rusted et al., 2000), and nicotine administration reduced the amyloid burden in APP transgenic mice
(Hellstrom-Lindahl et al., 2004; Nordberg et al., 2002). Third, a subset of nicotinic receptors, those containing α7 subunits, can interact with the Aβ peptide. In vitro, Aβ binds to α7 nAChRs and blocks the receptor, altering calcium homeostasis and potentially impairing neuronal signaling (Dineley et al., 2001, 2002a,b; Grassi et al., 2003; Pettit et al., 2001; Wang et al., 2000a,b). In APP transgenic mice, there are reports of decreased α7 nAChRs in older animals, which correlated with amyloid burden and cognitive impairments (Dineley et al., 2001, 2002a,b). Lastly, α7 nAChRs have been implicated in the inflammatory response. In human AD brain, reactive astrocytes co-localize with amyloid plaques and label for α7 nAChRs (Teaktong et al., 2003). There is also evidence that microglia can express α7 nAChRs (Shytle et al., 2004). These nicotinic receptors have also been studied in the peripheral immune system. Wang and colleagues demonstrated that α7 negatively regulates inflammation, as α7 knock out mice showed an enhanced inflammatory response to LPS challenge versus wild type mice (2003).

Based on the potential of α7 nAChRs to interact with Aβ, we further investigated this relationship in our model of LPS induced neuroinflammation (Herber et al., 2004c). We used several commercially available antibodies to detect α7 nAChRs by immunohistochemistry and Western blotting. We saw no changes in immunohistochemical labeling of neurons for α7 when comparing amyloid depositing mice (APP, APP:PS1) to nontransgenic mice, in contrast to previous reports (Dineley et al., 2001, 2002a,b). However, we confirmed the findings of Teaktong and colleagues by showing α7 nAChR immunoreactive astrocytes which co-localized with Congophilic
deposits in APP and APP:PS1 mice. In order to determine if this was a result of \( \alpha 7 \) interacting with \( \alpha \beta \), or part of a more general inflammatory response, we injected LPS into the hippocampus of several mouse models (nontransgenic, APP transgenic, and \( \alpha 7 \) knock out mice). This treatment resulted in substantial astrogliosis and immunolabeling by \( \alpha 7 \) antibodies as determined by immunohistochemistry, in all genotypes tested. These results were compared to Western blot, DNA, and RNA analysis and established the nonspecificity of the \( \alpha 7 \) antibodies. Recent reports have confirmed the nonspecificity of not only \( \alpha 7 \) but also \( \alpha 4 \) nAChR antibodies (Moser et al., 2004). Current work by Dineley and colleagues showed decreased soluble \( \alpha \beta \) in APP mice crossed with the \( \alpha 7 \) knockouts, thus there may be some role for \( \alpha 7 \) in Alzheimer’s disease, though determining the exact mechanisms will require rigorous testing protocols (Dineley et al., 2004).

The remaining chapters of this dissertation looked at the contribution of glia to the brain inflammatory response in order to gain insight from reactive gliosis and apply that information to the pathological processes of AD. Neurons, astrocytes, and microglia all contribute to the inflammatory response seen in AD, though the precise role of each cell type in this reaction is unclear.

In the earlier part of the twentieth century, Rio-Hortega proposed that microglia are the resident macrophages of the central nervous system (1932). Microglia are immunocompetent and act as mediators of innate immunity, but can also present antigen as part of the adaptive immune response (Streit, 2002). Similar to peripheral macrophages, microglial activation has both toxic and protective roles, thus these cells
are likely candidates for mediating inflammation, neurotoxicity, and/or Aβ removal in AD brain. Activated microglia can produce many neurotoxic species such as complement proteins, cytokines, prostaglandins, and reactive oxygen species (Akiyama et al., 2000). In 1996 Kreutzberg described various microglial activation states. Activated microglia undergo dramatic changes in morphology, and these changes are possibly linked to phagocytic versus antigen presenting functions. Hauss-Wegrzyniak and colleagues have also described a “bushy” phenotype that arises during chronic LPS infusion into brain ventricles (1998). Streit and colleagues (1999) have proposed the following phenotypes for reactive microglia, shown in Figure 23.
Figure 23. Microglial activation states. Neuronal injury can trigger injury signals which activate resting (ramified) microglia. The branches of these cells can become swollen (reactive/activated) or hyper-ramified (an intermediate stage). Microglia can also further activate and become phagocytic.
In general, the microglial inflammatory response to amyloid deposits is viewed as harmful (Akiyama et al., 2000). \textit{In vitro} studies have demonstrated the neurotoxic properties of both Aβ peptides as well as inflammatory mediators such as cytokines and reactive oxygen species (Canevari et al., 2004; Small et al., 2001; Walsh et al., 2002). However, \textit{in vivo} neurodegeneration due to either Aβ or inflammation has been difficult to demonstrate.

Intracranial administration of LPS has been used historically as an \textit{in vivo} model of neuroinflammation, activating both microglia and astrocytes. LPS is bound by the LPS binding protein which can then associate with CD14 and TLR4 to transduce a signal through the cell (Heumann & Roger, 2002; Thomas et al., 2002). Several pathways are activated including NFκB, resulting in gene transcription and the ultimate production of inflammatory mediators (Sen & Baltimore, 1986). The LPS signaling cascade is illustrated in Figure 24.
Figure 24. Lipopolysaccharide signaling cascades. Lipopolysaccharide (LPS) is bound in the circulation by LPS binding protein (LBP). This complex then interacts with cell membrane associated CD14 and toll like receptor 4 (TLR4) to transduce an intracellular signal. Various kinase cascades are induced (such as p38MAPK and IκB) leading to the activation of transcription factors including STAT, AP1, and NFκB. Down stream targets include cyclooxygenase (COX), nitric oxide synthase (iNOS), and superoxide dysmutase (SOD). Ultimately inflammatory mediators are produced such as prostanoids, nitric oxide (NO), tumor necrosis factor (TNF), and interleukins (IL).
Chapters 2 and 3 of this dissertation looked at the time course of glial activation after LPS administration into either nontransgenic mice or APP transgenic mice. Several markers of gliosis were examined including CD45, complement receptor 3 (CR3), Fc\(\gamma\) receptors, scavenger receptors, and GFAP. Both sides of the inflammatory response - toxic versus protective - were evaluated.

Brain inflammation begins with the glial response to insult. Once cells are activated, they can produce inflammatory agents which can be cytotoxic. LPS administration into both transgenic APP and nontransgenic mice led to acute and chronic microglial activation (Herber et al., 2004a,b). Two proteins, CD45 and CR3 were upregulated beginning as early as 24 hours after injection of LPS, and remained upregulated after 28 days. Resting and reactive microglia express these two proteins, and thus CD45 and CR3 are considered markers of general microglial activation. CD45 (leucocyte common antigen, LCA) is a protein tyrosine phosphatase. The endogenous ligand and the exact functioning of this protein in inflammation are unknown (Irie-Sasaki et al., 2003). Complement receptor 3 (CR3, CD11b, MAC-1) is a \(\beta_2\) integrin which mediates cell adhesion (Ehlers, 2000). In addition to the extracellular matrix, a prominent endogenous ligand for CR3 is complement component C3b1. Downstream activation pathways include MAP kinase activation and ultimately actin reorganization, promoting migration and phagocytosis. It is noteworthy that CR3 may also bind to LPS, thus activating the alternative pathway. In our studies, large increases in the levels of CD45, CR3, and the cytokines IL1\(\beta\) and TNF\(\alpha\) were seen after a single injection of LPS, indicating a significant inflammatory response. A spreading wave of inflammation was noted with the injected hippocampus responding first, then adjacent cortices and the
striatum, and ultimately the entire brain (including frontal cortex and brainstem) showed microglia expressing high levels of CD45 and CR3. A surprising finding was the continued expression of these two proteins in the hippocampus by microglial cells 28 days after LPS injection. This may indicate a state of vigilance by the microglia, with the cells primed for future action.

Changes in cell morphology at various time points after LPS injection was examined with CD45 and CR3 immunostaining. Untreated nontransgenic mice had microglia which were faintly stained for CD45/CR3 and had a ramified appearance with fine delicate processes. In untreated amyloid depositing mice (APP, APP:PS1), the microglia showed increased staining and had a bushy, reactive phenotype in association with deposits. After LPS administration, the microglia increased both CD45 and CR3 expression. Some had shorter, thicker, branched process, with a bushy shape, and were seen throughout the time course after LPS injection. Other cells were round or ameboid and were only transiently seen from 1-7 days after LPS. Though resting microglia can be distinguished from peripheral macrophages by their branched shape, round and/or ameboid microglia are impossible to discriminate from peripheral macrophages. Thus, in vivo it is difficult to determine which exact cell type is being evaluated because all macrophages express similar markers. It is also difficult to draw correlations from studies conducted in vitro. It is currently not possible to culture adult mouse microglia, thus neonatal or immortalized microglial cell lines are typically used (Yao et al., 1990; Colton et al., 1991; 1992a,b; Colton & Gilbert, 1993; Kopec & Carroll, 1998). Studies using adult versus neonatal mouse astrocytes however show vast differences between the two populations, and similar disparities can be assumed for microglia (Wyss coray et al.,
2003; Paul et al., 2004). Thus, though microglia clearly become activated, produce inflammatory mediators, migrate, proliferate, phagocytose, and present antigen in vitro, the same functions are not as clear in vivo.

Though a widespread, significant inflammatory response was seen, neuron death was not observed in any of our studies. This is different from the brain’s response to chronic LPS administration. Several publications by Wenk and Hauss-Wegrzyniak and colleagues over the past 15 years have demonstrated the effects of chronic infusion of LPS into the fourth ventricle of rats (Hauss-Wegrzyniak et al., 1998a, b; 2000; 2002; Willard et al., 1999). Their protocol led to significant astro- and microgliosis as well as forebrain cholinergic neuron death. In contrast, acute LPS injection into the brain parenchyma such as the anterior cortex and hippocampus does not triggered cell death, as demonstrated by our lab and others (DiCarlo et al., 2001; Herber et al, 2004a,b; Kim et al., 2000). However the substantia nigra, a part of the dopaminergic system implicated in the pathogenesis of Parkinson’s disease, is susceptible to the degenerative inflammatory effects of acute LPS administration (Castano et al., 2002; Kim et al., 2000). In our own studies, acute intrahippocampal LPS administration did not cause detectable neurodegeneration as determined by cresyl violet and fluorojade staining, nor was synaptic dysfunction detected by synaptotagmin levels, regardless of dose, time, or genotype examined (Herber et al., 2004a,b). LPS injection induced NFκB expression (unpublished data) which has been linked to neuronal survival, and thus may be protective under the conditions of our protocol (Kassed et al., 2004). The only conditions which caused neuron death were co-injections of LPS and interferon gamma
It is possible that the presence of both endogenous and exogenous inflammatory mediators are necessary to kill cells. The protective aspects of microglial activation include neurotrophic support, as well as clearance of cellular debris, foreign substances, and microbial components. Microglia have demonstrable phagocytic functions, similar to peripheral macrophages (Kalmar et al., 2001; Yao et al., 1990; Czapiga & Colton, 1999). In our studies, stimulation of the innate immune system by intrahippocampal injection of LPS led to time-dependent reductions in Aβ levels by as much as 70% (Herber et al., 2004b). The reductions in Aβ were transient, occurring between 3 and 14 days after LPS injection, then returning to baseline levels after 28 days. Diffuse Aβ material, but not compacted Congophilic deposits, were decreased after LPS treatment (it is interesting to note that stimulating microglia using an Aβ antibody reduced both diffuse and compact deposits in a similar protocol; Wilcock et al., 2003; 2004a,b). The time course of Aβ reductions after LPS injection was closely related to the expression of microglial phagocytic markers and changes in cell morphology. We therefore believe that the microglia are removing the Aβ, thus performing their protective phagocytic function.

Other research groups have administered LPS to accelerate Aβ deposition, activate glia, and trigger neurotoxicity. The experimental conditions of reports vary including the type and age of transgenic animals used; type, dose, and route of administration of LPS; and post- injection survival time. In some cases amyloid deposition was triggered (Qiao et al., 2001; Sheng et al., 2003; Sly et al., 2001), while in others amyloid clearance resulted (DiCarlo et al., 2001; Quinn et al., 2003). When considering the species of Aβ1-40 and Aβ1-42, the main indicator of Aβ reduction seems to
be closely related to plaque load and survival time. Aged animals with significant amyloid deposits, surviving several days post injection, showed clearance of Aβ deposits after LPS treatment. Compact plaques detected by either Congo red or thioflavin-S staining were inconclusively affected.

In our studies, we examined markers of a phagocytic microglial phenotype including Fcγ and scavenger receptors at various time points after LPS injection. Resting microglia do not express appreciable levels of Fcγ receptors, though some plaque associated microglia in APP mice do stain positively for the receptors. After LPS injection, both protein (FcγRII/III) and mRNA (FcγRII) levels increased, peaked between 24-72 hours, and then returned to low basal levels after a week. Fc receptors are traditionally linked to adaptive immune responses, with the Fc portion of the antibody:antigen complex as the ligand (reviewed in Gessner et al., 1998). The bound complex can then be internalized, processed, and antigen presented via MHCII. The ligand for Fcγ receptors is IgG and there are three classes, with class I a high affinity, and classes II and III low affinity receptors. Class I and III are activating; class II is inhibitory. The various functions of Fcγ receptors as listed in the Gessner review include, “clearance of antigen/antibody immune complexes, regulation of antibody production, enhancement of antigen presentation, antibody-dependent cell-mediated cytotoxicity, phagocytosis, degranulation, and activation of inflammatory cells.” There is evidence that peritoneal macrophages and B-cells express FcγRII after treatment with LPS, even in the absence of antibody:antigen complexes (Amigorena et al., 1989). Microglia and bone marrow derived macrophages can also be induced to express Fc receptors after LPS or interferon gamma treatment (Keller et al., 1994; Loughlin et al., 1993).
experiments described herein, the innate immune system is involved, but we would not
expect antibody production as part of an acquired immune, thus Fc expression might be
part of a general inflammatory response, priming the cell for phagocytosis should an
adaptive response be mounted.

Scavenger receptors A and B are also involved in the recognition of LPS by the
innate immune system and can mediate clearance of bacteria and viral components. In
our studies, only a brief, mild increase in SRB mRNA was seen at 6 hr after LPS
injection. We were unable to confirm SRB expression by immunohistochemistry as
available antibodies showed nonspecific staining patterns. In contrast, a significant,
hirty fold increase in SRA was seen both in mRNA and protein levels. Resting microglia
did not express appreciable levels SRA, nor did plaque associated microglia in untreated
APP mice. After LPS injection, SRA increased with widespread expression, peaked
between 24-72 hours, and then returned to low basal levels after a week. The cell
population staining for SRA appeared to be microglia/macrophages based on their
morphology, rather than astrocytes.

Scavenger receptors are traditionally linked to lipid metabolism and innate
immune responses (Husemann et al., 2002; Kreiger et al., 2001; Febbraio et al., 2001;
Platt and Gordon, 2001). They are also called pattern recognition receptors as they
nonspecifically recognize many bacterial and viral components, usually based on the
presence of polyanionic ligands. Both microglia and astrocytes can express various
scavenger receptors, depending upon age and activation state of the cell. Scavenger
receptors A and B have been implicated in AD with increased expression in glial cells
surrounding amyloid deposits (Coraci et al., 2002). We were interested in SRA
specifically because it has been shown to bind both LPS and Aβ, though other scavenger receptors can also bind Aβ (Husemann et al., 2002). Based on our results, we propose that LPS induced expression of scavenger receptors that could then bind Aβ and mediate removal. A diagram constructing the results from chapters 2 and 3 is found in Figure 25.
Figure 25. Mechanisms of LPS stimulated inflammation and Aβ removal. This cartoon is a composite of various stained sections. A cresyl violet stain of the neurons in the dentate gyrus (upper left corner) and portions of CA3 (lower left corner) are the backdrop of the figure (the cells are stained blue). Aβ deposits are stained brown, with compacted, Congophilic deposits stained red. A reactive astrocyte (stained for GFAP) is shown in the upper right corner. Microglia are stained for CD45 and are black, shown transitioning among various activation states. LPS injection increases levels of the cytokines IL1β and TNFα, as well as cell surface receptors CD45, CR3, SRA, and FcγRII/III. The graph in the upper left corner illustrates the changes over time in CD45/CR3 (purple), SRA and FcγRII/III (green) and Aβ (orange).
The final chapter of this dissertation sought to further confirm that microglia are involved in the LPS-stimulated removal of Aβ. We used dexamethasone co-treatment with LPS intrahippocampal injection, thereby blocking microglial activation. Dexamethasone is a potent glucocorticoid anti-inflammatory agent with similar physiological activities as cortisone (Hardman et al., 2001). Dexamethasone is bound in the circulation by corticosteroid binding globulin. Dexamethasone can then transverse the cell membrane as it is hydrophobic. Once inside the cell, dexamethasone binds to the glucocorticoid receptor in the cytosol, releasing inhibitory heat shock proteins from the glucocorticoid receptor allowing it to translocate to the nucleus. Glucocorticoid receptor:dexamethasone complexes can then antagonize NFκB response elements (as well as others), thereby inhibiting the inflammatory response.

In this final study, LPS induced the prototypical inflammatory response discussed previously. CD45, CR3, and IL1β levels were increased, as well as SRA and Fcγ receptors. Concurrent with this response, we demonstrated the expected decrease in the diffuse Aβ load, with no effect on compact deposits. Dexamethasone co-treatment significantly inhibited CD45, CR3 and IL1β levels. The reductions in diffuse Aβ caused by LPS were also inhibited by dexamethasone co-treatment, thus implicating the microglial response in the removal process. However, neither FcγR nor SRA were inhibited by dexamethasone co-treatment. These findings were confirmed by both immunostaining and mRNA analysis where possible.

Dexamethasone’s ability to inhibit reactive microgliosis is well documented. Our lab has previously demonstrated that CD45 levels (induced by intracranial injection of Aβ antibodies into APP mice) were significantly inhibited after dexamethasone treatment.
(Wilcock et al., 2004a). Others have shown that dexamethasone inhibits IL1β after either LPS or Aβ intraventricular injection into nontransgenic mice (Szczepanik & Ringheim, 2003). Thus, our findings that dexamethasone inhibited CD45, CR3, and IL1β and also subsequently prevented the removal of Aβ were expected. These data provide evidence that general microglial activation, as demonstrated by CD45 and CR3 levels, is required for the removal of Aβ in our protocol.

The lack of inhibition of Fc and scavenger receptors in the LPS-dexamethasone co-treated mice was an unexpected result. There is evidence to support these findings. Of all the markers examined, only FcγR class II have an identified a glucocorticoid response elements (GRE) in the promoter (Hogarth et al., 1991). In this case, the Fc receptor is inhibitory and the GRE is stimulatory, thus we would expect an increase in the levels of the class II receptor under dexamethasone- treated conditions. Sivo and colleagues reported similar results where interferon gamma- stimulated FcγRII/III expression, and was not inhibited by dexamethasone treatment (1993). A review of the literature did not reveal any data concerning dexamethasone’s effects on SRA expression. Taken together, the continued expression of FcγR and SRA after dexamethasone treatment indicates that expression of markers associated with phagocytosis are not sufficient to effect removal of Aβ in our protocol. Figure 26 illustrates our findings to date.
Figure 26. Mechanisms of LPS stimulated inflammation and Aβ removal, and inhibition by dexamethasone. This cartoon is a composite of various stained sections (details provided in Figure 25). LPS injection increases levels of the cytokines IL1β and TNFα, as well as cell surface receptors CD45, CR3, SRA, and FcγRII/III. Dexamethasone co-treatment inhibited IL1β, CD45, and CR3, and also prevented Aβ reductions, but had no effect on SRA or FcγR levels. The graph in the upper left corner illustrates the changes over time in CD45/CR3 (purple), SRA and FcγRII/III (green), and Aβ (orange).
Our current hypothesis is that microglia are removing the diffuse Aβ in our in vivo model. This idea is supported by the finding that dexamethasone inhibited microglial activation and Aβ removal. There are other possible explanations for dexamethasone’s action in our model. The three experimental groups evaluated in our final study were a) saline injected controls, b) LPS injected, and c) LPS injected plus dexamethasone treated APP mice. We did not evaluate dexamethasone-only treated mice; we were looking for the drug’s effect on LPS-induced inflammation, not dexamethasone’s effect on amyloid burden. There are no published studies looking at glucocorticoid administration to amyloid depositing mice in the absence of other stimulating agents. Human trials with prednisone did not ameliorate symptoms (Aisen et al., 2000). In vitro, neither prednisone nor dexamethasone lowered Aβ (Sagi et al., 2003). NSAIDS, which can lower Aβ in vitro and in long term studies with mouse models, do not have reproducible effects after short term treatment (3-5 days; reviewed in Gasparini et al., 2004). Therefore, it is unlikely that in the short time frames studied herein (3 and 7 days) dexamethasone would exert an appreciable effect on brain amyloid burden. However, it cannot be ruled out that the effects we saw here were due to the potential for dexamethasone to increase Aβ burden thus negating the effects of LPS. Alternatively, dexamethasone can decrease the permeability of the blood brain barrier, which may have prevented the flux of Aβ from the brain to the periphery (Romero et al., 2003). Future studies could evaluate dexamethasone’s effect on amyloid deposition in the absence of inflammation, as well as the effects of other anti-inflammatory drugs on microglial activation and Aβ reductions.
Our primary hypothesis regarding the removal of Aβ centered on the scavenger receptors. Based on our results, we proposed that LPS induced expression of scavenger receptors that could then bind Aβ and mediate removal. However, we found that SRB was not highly upregulated, and that SRA expression persisted in the absence of Aβ removal. Therefore, a working hypothesis regarding LPS-stimulated reductions in Aβ can be proposed: It is not the mere presence of the LPS molecule, nor is the upregulation of receptors involved in phagocytosis sufficient for Aβ removal. Rather, general cell activation is required. Thus, a phagocytic cell must not only bind Aβ (by various receptors) but must also be capable of engulfing the material (via general cell activation).

There are several cell surface receptors grouped under the general classification of scavenger receptors which can bind Aβ, including SRB, CD36, SRA, CD68, receptor of advanced glycation end products, and lipoprotein receptor related protein (Peiser et al., 2002; Huseman et al., 2002). SRB clearly interacts with Aβ and can mediate microglial activation (Coraci et al., 2002; Moore et al., 2002; El Khoury et al., 2003). There is some confusion as to the identity of SRB and CD36 (a class B type receptor). They are the focus of separate lines of research, but the sequences listed in Genbank are identical, and the nomenclature overlaps in the literature. For the work described herein, we used the sequence for murine CD36, and have referred to it as SRB throughout. Wyss-Coray and colleagues have shown that blocking SRB1 had no effect on astrocyte-mediated Aβ clearance (2003). Whether there is truly a difference between CD36 and SRB, and the potential roles for either receptor in LPS stimulated Aβ removal remains to be elucidated. Future studies could block scavenger receptors with fucoidan, or specific antibodies, to
further evaluate their role in our model. Scavenger receptor knockout mice for both CD36 and SRA are also available and could be bred with APP mice to test subsequent effects on LPS mediated Aβ removal.

CD68 (macrosialin) is another scavenger receptor implicated in the pathogenesis of AD. The endogenous ligands for CD68 are modified lipoproteins and it is commonly referred to as a late endosomal or lysosomal protein, though cell surface expression also occurs (Ramprasad et al., 1996; Holness et al., 1993). It has been shown to be upregulated in AD brain and in APP mice (Bornemann et al., 2001; Sasaki et al., 2002). We have briefly examined CD68 immunostaining in our model and found both plaque-associated microglia as well as LPS-induced widespread expression of CD68 (unpublished data). This protein may be marking a phagocytic population and deserves further investigation.

The receptor for advanced glycation end products (RAGE) has been intensely investigated in AD (Zlokovic et al, 2004). Endogenous ligands for RAGE include nonenzymatically glycated adducts, beta-pleated sheet structures, and Aβ. Rather than triggering Aβ removal via endocytosis, stimulation of RAGE leads to prolonged cell activation (Schmidt et al., 2001). RAGE is upregulated in AD brain, particularly around plaques, and has been proposed to mediate the flux of Aβ from the periphery into the brain, thereby altering the balance between deposition and removal. Lipoprotein receptor related protein (LRP) is thought to act as counterpoint to RAGE by mediating flux of Aβ out of the brain into the periphery with APOE mediating binding to the receptor. The main ligands for LRP are modified lipoproteins, but this receptor can also bind bacterial and complement components. In vitro models have shown that LPS decreased
macrophage LRP expression, whereas dexamethasone increased LRP levels (Laithwait et al., 1999; Marzolo et al., 2000). The effects \textit{in vivo} remain to be elucidated thus LRP is an attractive target for investigation in our model of LPS mediated removal of Aβ. Future studies to further elucidate the Aβ removal described herein would evaluate each of these proteins under both LPS and dexamethasone treatment conditions.

An alternative explanation is that scavenger receptors or phagocytosis in general may not be involved at all. Other mechanisms of Aβ removal include non scavenger receptor-mediated phagocytosis, decreased APP processing, increased proteolytic degradation of Aβ, or contributions from the periphery in clearance.

Integrins mediate cell adhesion to both the extracellular matrix and to other cells, triggering intracellular signaling. The α6β1 integrin is expressed by macrophages and binds Aβ \textit{in vitro} as part of a trimolecular complex and mediates phagocytosis (Bamberger et al., 2003; Koenigsknecht & Landreth, 2004). The trimolecular complex includes α6β1, CD36, and CD47 (an integrin associated protein). Though we investigated CD36 (SRB) in our model, we have not yet evaluated the roles of α6β1 or CD47 and this remains a potential route of LPS mediated Aβ removal. CR3 is another integrin (β2) and is the receptor for complement component C3. CR3 is a pattern recognition receptor which can bind LPS (Ehlers et al., 2000), though no data on its ability to bind Aβ was found after a literature search. Complement protein C3 is a major component of the alternative pathway. Wyss-Coray and colleagues have shown that inhibiting C3 led to increased Aβ deposition in transgenic mice (2002). In our studies, CR3 was upregulated by LPS, and inhibited by dexamethasone, mirroring the effects on
Aβ removal. Therefore CR3 could be directly involved in Aβ reductions in our model. Future studies could inhibit the integrins using invasin and evaluate subsequent effects on Aβ levels.

Several metalloproteinases have been shown to degrade Aβ, including neprilysin (NEP), insulin degrading enzyme (IDE), and matrix metalloproteinase 9 (MMP-9). NEP is capable of degrading many circulating peptides including enkephalin, substance P, and Aβ. NEP polymorphisms have been linked to increased risk of AD (Sakai et al., 2004), and transgenic mouse models have confirmed that overexpression of NEP can reduce brain Aβ levels (Mohajeri et al, 2004; Iwata et al., 2004). We conducted a preliminary investigation to determine NEP levels after LPS injection in our model. We did not find any significant changes in the immunostaining patterns (unpublished findings). Kaneko and colleagues have evidence that LPS decreases NEP levels in neutrophils, though effects on other macrophage populations are unknown (2003). Another enzyme, IDE, degrades both insulin and Aβ. Based on the relationship between diabetes mellitus and AD, an increased risk for AD was found with certain variants of the IDE gene, and IDE activity, levels, and mRNA are decreased in AD brain (reviewed in Zhao et al., 2004). A search of the literature did not reveal data reporting the effects of LPS on IDE levels, and we have not looked in our studies. A separate class of metalloproteinases is associated with the extracellular matrix, and has been implicated in plaque formation in cardiovascular and Alzheimer’s diseases. MMP-1, -3, and -9 are capable of degrading Aβ, and the levels of these enzymes are altered in AD brain (Leake et al., 2000; Helbecque et al., 2003; Yoshiyama et al., 2000). Woo and colleagues recently showed that LPS can induce expression of MMP-9 in macrophages (2004), and thus may be
partially responsible for the reductions in Aβ in our model. Future studies should include evaluation of these enzymes in our model.

One simple explanation for the decreased Aβ load in our model is decreased levels of APP or its processing. In an attempt to answer this question, we immunostained for APP and also measured mRNA levels after LPS administration (data not shown). The immunostaining pattern was found to be nonspecific, and no significant changes in APP mRNA levels were found in response to LPS. Thus we do not believe there were large changes in APP levels which could account for the up to 70% decreases in Aβ seen in our studies. In general, most research regarding the brain’s response to insult indicates that APP levels increase, and presenilin is also induced (Pennypacker et al., 1999; Brugg et al., 1995; Siman et al., 1989; Wright et al., 1999; Sheng et al., 2003). In other studies, LPS was shown to increase neuronal levels of Aβ species, though the researchers did not specifically examine the secretases (Sheng et al., 2003; Sly et al., 2001). The activity or levels of alpha, beta and/or gamma secretases may be altered after LPS injection, which could account for the decreased levels of Aβ in our model. We did not measure the secretases, thus such studies represent future lines of investigation.

Another unexplored option in the LPS-mediated Aβ removal is the transcription factor peroxisome proliferator-activated receptor (PPARγ). PPARγ is traditionally associated with lipid and insulin metabolism and is targeted in therapies for Type II diabetes, but also has potent anti-inflammatory actions. Camacho and colleagues recently reported in an in vitro assay that the PPARγ agonists rosiglitazone and pioglitazone reduced Aβ secretion from APP transfected HEK cells (2004). However, our
own lab failed to demonstrate reductions in amyloid burden after pioglitazone administration to transgenic mice (Paul Jantzen et al., unpublished results.) In another study using rats, PPARγ was down-regulated by peripheral LPS treatment (Ohata et al., 2004). However, the effects of intracranial administration of LPS are unknown and thus PPARγ represents another potential mechanism mediating Aβ reductions.

Contributions of the peripheral immune system to the reductions in Aβ in our model cannot be ruled out. There is evidence of peripheral blood-borne cells in the brains of our LPS injected mice as seen in the small round cells seen between 24 and 72 hours after LPS injection. As much as 80% of these cells may be recruited monocytes (Montero-Menei et al., 1996). In order to rule out peripheral cells as mediators of Aβ removal, we could irradiate the mice or chemically ablate their bone marrow cells, and then inject LPS and monitor subsequent effects on Aβ burden. Another potential mechanism of Aβ reductions can be found in the peripheral sink hypothesis (Matsuoka et al., 2003). According to this line of thinking, Aβ levels can be reduced by creating a concentration gradient down which Aβ would move out of the brain and into the periphery. LPS has been shown to disrupt the blood brain barrier and may therefore alter Aβ trafficking (Nonaka et al., 2005). We did not evaluate the levels of Aβ in the periphery and therefore do not know the contribution of any potential sink in our model. Future studies could collect blood from the mice after treatment and evaluate the levels of Aβ.

The majority of the markers we used to evaluate gliosis in our model are macrophage specific. Astrocytes may also contribute to the removal of Aβ seen herein.
Astrocytes normally perform homeostatic functions but also react to amyloid deposits in the brain. Astrocytes are capable of releasing inflammatory mediators including complement proteins, cytokines, prostaglandins, and reactive oxygen species (Akiyama et al., 2000). These cells are spatially associated with amyloid and may be forming a glial scar around the deposit, though astrocyte phagocytic functions are also possible (DeWitt et al., 1998; Kurt et al., 1999; Mandybur et al., 1990; Wegiel et al., 2000). One of the most compelling studies linking astrocytes to amyloid removal was demonstrated by Wyss-Coray and colleagues in 2003. They coated brain slices from APP transgenic mice with adult mouse astrocytes. A significant 40% reduction in the total amyloid burden was seen after 24 hour incubation. Fucoidan and polyinosinic acid significantly blocked Aβ removal, implicating scavenger receptors in the process. In our studies, the only true astrocyte marker evaluated was GFAP. LPS induced a significant increase in GFAP in all of our studies, though the reaction seemed to be transient. In vivo, it is difficult to separate individual populations of cells and their activities, thus some dual labeling may be necessary once the receptors/mechanism of Aβ removal is discovered, in order to confirm whether it is microglia and/or astrocytes involved in this process.

Assuming that the amyloid hypothesis is correct, removing Aβ should ameliorate the symptoms and/or progression of AD. Strategies aimed at stimulating the immune system to remove Aβ are at the forefront of AD research. There is evidence from the human vaccine trial that treatment successfully decreased amyloid load (Schenk et al., 2004). An immune/inflammatory reaction was seen during the trial, which led to meningoencephalitis in rare cases, even though many other patients experienced cognitive stability and possible improvement due to the vaccine. In transgenic mice, both
passive and active immunization against Aβ led to microglial activation in the process of lowering Aβ burden and improving cognitive performance (Wilcock et al., 2001, 2003, 2004a,b). Our own results with stimulation of the innate immune system with LPS yielded similar results: activation of microglia resulting in reductions in Aβ. In none of the laboratory trials was neurodegeneration triggered by the inflammatory response. Taken together, one might conclude that some level of inflammation in AD is actually beneficial and is responsible for maintaining a balance between amyloid deposition and removal.

Future studies would aim to characterize the activation state of microglia by either stimulating them with various agents or inhibiting specific aspects of the inflammatory response. Though LPS primarily signals through TLR4, an interesting approach would be to activate the cells via other toll-like receptors, and then examine differential microglial activation while monitoring effects on amyloid burden. In our studies, the inflammatory reaction seen after innate immunity stimulation was blocked by dexamethasone, and also prevented Aβ removal. Targeted anti-inflammatory or anti-oxidant treatments using compounds such as nitro-flurbiprofen, celecoxib, pioglitazone, or melatonin could be useful in identifying the degree of cell activation required for phagocytosis. It is increasingly evident that the inflammatory response, particularly modulated by microglia, is very complex. Therapeutic opportunities await analysis of the different stages of microglial activation, their function, morphology, and protein expression during both the chronic response to amyloid as well as during phagocytic removal of the deposits.
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