PPARγ ACTIVATION RAPIDLY AMELIORATES AMYLOID PATHOLOGY AND RESTORES COGNITION IN A MOUSE MODEL OF ALZHEIMER’S DISEASE

By

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Abstract

By

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Alzheimer’s disease (AD) is a chronic neurodegenerative disease characterized by the progressive loss of cognition and memory. The pathological hallmarks of AD include extracellular amyloid deposits and intra-neuronal neurofibrillary tangles. Progression of the disease is associated with a disruption of Aβ homeostasis and accumulation and deposition of Aβ in the brain parenchyma, initiating a robust microglial-mediated immune response that leads to the production of pro-inflammatory cytokines, chemokines and reactive nitrogen and oxygen species which are deleterious to the CNS. Despite the abundance of activated microglia surrounding plaques, they are inefficient in clearing fibrillar Aβ deposits. It is thought that activating mechanisms that facilitate Aβ clearance will alleviate disease related pathophysiology and be of great therapeutic utility for the treatment of Alzheimer’s disease.

The peroxisome proliferator activated receptor-γ (PPARγ), is a ligand activated nuclear receptor, which regulates lipid and glucose homeostasis in the body and exhibits potent anti-inflammatory actions. Activation of PPARγ has been shown to reduce brain Aβ levels and ameliorate AD related cognitive deficits in animal models of AD. However, the mechanisms through which PPARγ attenuates AD related pathophysiology have yet to be elucidated. We have shown that activation of another nuclear receptor, LXRα, facilitates the proteolytic degradation of Aβ in an ApoE-dependent mechanism.
through induction of target genes, ABCA1 and ApoE. ABCA1 regulates cholesterol efflux via lipidation of ApoE and this process augments the degradation of soluble Aβ species. Importantly, PPARγ activation induces the expression of LXRα and its targets ApoE and ABCA1, metabolically linking these pathways.

We demonstrate that PPARγ activation using the synthetic agonist, Pioglitazone (Actos™) enhances the proteolytic degradation of Aβ in microglia and astrocytes by utilizing the PPAR-LXR-ApoE linked pathway in the brain. PPARγ mediated intracellular degradation of Aβ is dependent on expression of ApoE and LXR. Furthermore, an acute (9 day) treatment of APP/PS1 mice with pioglitazone, rapidly increased brain levels of ABCA1 and ApoE, decreased amyloid deposition and ameliorated AD-related cognitive deficits. The reduction in plaque deposition was paralleled with an increase in amyloid-laden microglia and astrocytes in the parenchyma of treated animals. Significantly, pioglitazone treatment of APP/PS1 animals polarized CNS microglia from a “classical” to an “alternative” activation state, reducing glial activation, re-engaging the phagocytic machinery and facilitating the clearance of fibrillar Aβ deposits. Together our data demonstrates an important role for PPARγ activation in facilitating the clearance of both soluble and fibrillar species of Aβ and provides a mechanistic explanation for how PPARγ agonists reduce AD-related pathophysiology.
Chapter 1

Introduction
Alzheimer’s Disease

Alzheimer’s disease (AD) is the leading cause of dementia in the elderly today. This disease was first characterized by Dr. Alois Alzheimer in 1906, whose patient, a middle-aged woman, Auguste Deter, had developed memory deficits and progressive loss of cognitive abilities. After her death, Dr. Alzheimer performed post-mortem analysis of her brain and identified the key pathological features of AD, amyloid plaques and neurofibrillary tangles, that are used to this day to diagnose the disease (Moller and Graeber, 1998). Amyloid plaques are extracellular deposits of Aβ peptides which can be most readily visualized in autopsy sections stained with silver impregnation techniques or upon staining with lipophilic dyes such as Congo Red and thioflavin S. Similar staining can often be observed in the vessel walls of capillaries or larger blood vessels, a pathological feature known as cerebral amyloid angiopathy that accompanies AD. Plaques are generally found throughout the cerebral cortex and the hippocampus. Neurofibrillary tangles are found within neurons of the cerebral cortex and hippocampus and consist of insoluble intracellular fibrils that are composed of hyperphosphorylated forms of tau, a microtubule-associated protein (Wood et al., 1986, Yen et al., 1987). The microtubules are essential for axonal transport and the structural stability of neuronal processes. Therefore, it is believed that impaired axonal transport contributes to neuronal degeneration that typifies the disease. Over a hundred years have passed since the initial
characterization of this disease, however, the mechanisms of actions underlying disease pathogenesis still remain elusive.

There are currently 5 million people with AD and it is estimated that over 16 million persons will be afflicted with this disease by the year 2050 (2010). Age is the single largest risk factor for developing AD and about 50% of the population over the age of 85 has Alzheimer’s disease (Gorelick, 2004). Other risk factors include, gender, possession of the Apoe4 allele, head trauma and loss of estrogen (Breteler et al., 1992, Payami et al., 1996, Kim et al., 2009). Once diagnosed the disease progresses over an average of 8 years and is clinically characterized by progressive cognitive decline, memory loss, loss of motor skills and hallucinations. The annual cost of treatments for AD is now $170 billion. At this rate, AD is reaching epidemic proportions and treatments aimed at prevention or treating this disorder are urgently needed.

Currently, AD therapies are aimed at ameliorating symptoms associated with the disease, and there are no drugs available that target the underlying pathology of Alzheimer’s disease. These drugs operate by regulating levels of neurotransmitters the brain. They do this namely by preventing their degradation. The problems with these drugs is that firstly, they are beneficial to only a small population of patients afflicted with the disease and secondly, their effects are quite transient. It is thought that delaying the onset of the disease by just five years could reduce the number of people afflicted with AD by 50%. Thus, new therapeutics are in great need.

**Amyloid Precursor Protein and Aβ Production**
Alzheimer’s disease is a chronic neurodegenerative disease characterized by the progressive deposition of the amyloid beta (Aβ) in the parenchyma of the brain. Glenner and Wong first identified Aβ, a 4 KiloDalton (KD) protein, as the major constituent of amyloid plaques in patients with AD and Down’s syndrome in 1984 (Glenner and Wong, 1984a, b). It was later determined that Aβ was derived from a larger type 1 transmembrane protein called the amyloid precursor protein (APP), whose physiological function still remains unknown. The APP gene is located on chromosome 21, contains at least 19 exons and is preferentially expressed in neurons.

Processing of APP can occur through one of two different mechanisms. Nonpathogenic processing of APP occurs when the APP protein is cleaved first by the α-secretase, releasing the N-terminal fragment (sAPPα) and leaving behind a membrane-bound C-terminal fragment (Buxbaum et al., 1998, Asai et al., 2003). This C-terminal fragment is then cleaved by γ-secretase into a soluble N-terminal fragment (p3) and the APP intracellular domain (AICD). The γ-secretase complex is composed of 4 different membrane associated proteins including nicastrin, Aph-1, presenilin and Pen-2 (Francis et al., 2002, Fraering et al., 2004). Pathogenic processing of APP occurs when APP is first processed by the BACE1 (β-secretase), a transmembrane aspartic protease, leading to the production of a soluble N-terminal fragment (sAPPβ) and a membrane bound C-terminal fragment (CTFβ). The γ-secretase then acts upon CTFβ yielding the production of Aβ (which varies in length between 38 and 43 amino acids) and AICD (Vassar et al., 1999, Selkoe, 2001) (Figure 1).

Genetically inherited forms of AD arise from mutations in APP that are clustered in the vicinity of the beta and gamma secretase sites or within the gamma secretase itself,
favoring Aβ generation (Tanzi and Bertram, 2005). These cases of early onset Alzheimer’s disease (EOAD) manifest their symptoms between 30 and 60 years of age. Currently, 140 mutations in PSEN1, 10 in PSEN2 and 30 in APP have been identified as causing EOAD (Tanzi and Bertram, 2005). Similarly, Down’s syndrome patients, who have an extra copy of chromosome 21 and therefore an extra copy of APP, develop AD between the ages of 40 and 50 (Wisniewski et al., 1985). Many of these mutations have been valuable in facilitating our understanding of AD and the prominent role of Aβ in disease pathogenesis. These mutations have now been exploited for the development of a number of different transgenic mouse models of AD, allowing us to extensively study this disease.

**Late Onset Alzheimer’s Disease**

While genes associated with EOAD have broadened our understanding of AD they account for a very small fraction of AD cases. Over ninety percent of AD cases are “sporadic” and manifest their symptoms later in life. While the mutations associated with EOAD are fully penetrant and can be traced in a family tree, the genetics associated with late-onset Alzheimer’s disease LOAD are far more complex (Bertram et al., 2010). While genome-wide linkage studies have provided strong evidence for the association of many genes with AD, the Apolipoprotein E4 (apoE4) allele is the single most significant risk factor for AD (Schmechel et al., 1993, Strittmatter et al., 1993). Moreover, metabolic diseases such as diabetes, metabolic syndrome, atherosclerosis and other types
of cardiovascular disease are associated with an increased risk for LOAD (Martins et al., 2006, Razay et al., 2007).

**Aβ Homeostasis**

A substantial body of literature supports the centrality Aβ homeostasis in disease progression. The Aβ peptide is generated by the sequential cleavage of the amyloid precursor protein (APP) by the beta and gamma secretases, resulting in the generation of peptides 40 or 42 amino acids in length (Sisodia and Price, 1995). Aβ_{42} contains two extra hydrophobic residues at its C-terminus than Aβ_{40}, promoting fibrillogenesis. Furthermore, the β-pleated sheets located at the C-terminal of the protein, promote aggregation and seed plaque cores. Aβ, is a self-associating protein can sequentially deposit into dimers, trimers, large oligomers, Aβ derived diffusible ligands (ADDLs), protofibrils and then fibrils (Standridge, 2006). These fibrils then deposit into both diffuse and dense-core plaques, principally in the cortex and hippocampus (Figure 2). It has been postulated that soluble or small oligomeric forms of Aβ have deleterious effects in the brain, inducing impaired synaptic function and promoting neuronal degeneration (Lacor et al., 2007). The development of dense-core amyloid plaques is associated with a robust immune response mediated by microglial cells.

According to the amyloid hypothesis, it is the gradual accumulation of Aβ in the brain that leads to disruptions in brain homeostasis and promotes pathogenesis in AD. Thus, enhanced removal of Aβ from the diseased brain may alleviate symptoms associated with the disease. Clearance of Aβ peptides from the brain can occur through
efflux of Aβ through the blood brain barrier (BBB) or the proteolytic degradation of the peptide within the brain by cells or by extracellular proteases (Tanzi et al., 2004).

Many cell types have been documented to take up Aβ in the CNS, including microglia, astrocytes and vascular endothelial cells (Tanzi et al., 2004). Uptake can occur through fluid-phase pinocytosis or phagocytosis of fibrillar Aβ species (Paresce et al., 1997a, Chung et al., 1999, Mandrekar et al., 2009). Microglia have been the most dominantly studied cell-type for this process, however, recent studies have shown that astrocytes may play a large role in the removal of Aβ from the brain as well (Funato et al., 1998, Wyss-Coray et al., 2003, Koistinaho et al., 2004, Fuller et al., 2009).

A plethora of proteases have been reported to degrade Aβ in vitro, including neprilysin (NEP), insulin degrading enzyme (IDE), plasmin, angiotensin converting enzyme (ACE), endothelin converting enzyme 1 (ECE1), matrix metalloproteases 2, 3 and 9 (MMP2, MMP3, MMP9), the mitochondrial metalloprotease PreP and EC 3.4.24.15 (TOP) (Papastoisitsis et al., 1994, Backstrom et al., 1996, Iwata et al., 2001, Eckman et al., 2003, Farris et al., 2003, Melchor et al., 2003, Morelli et al., 2003, Hama et al., 2004, Hemming and Selkoe, 2005, Falkevall et al., 2006, Yin et al., 2006). Genetic analysis have also demonstrated a linkage of these proteases to AD (Miners et al., 2008). However, NEP, IDE, ECE, plasmin have been shown to exert positive effects on amyloid pathology in vivo as well (Iwata et al., 2001, Eckman et al., 2003, Farris et al., 2003, Liu et al., 2009).

Efflux of soluble Aβ from the brain into the periphery can occur through a number of different mechanisms. The low density lipoprotein receptor-related protein-1
(LRP1) is the most well-studied receptor for facilitating the clearance of soluble Aβ (Zerbinatti and Bu, 2005). LRP1 is an ApoE receptor and is expressed at high levels in the CNS and Aβ has been shown to bind directly to LRP1 or through its association with ApoE (Deane and Zlokovic, 2007). LRP1 is postulated to mediate the efflux of Aβ across the brain capillary endothelium (Deane et al., 2004). P-glycoprotein (PgP), is found in the brain endothelia and has also be implicated in transported Aβ across the BBB (Cirrito et al., 2005, Deane and Zlokovic, 2007). AD transgenic mice deficient in PgP have been shown to clear Aβ at half the rate of wildtype littermates and have increased plaque burden (Cirrito et al., 2005).

**Microglial Dynamics in a Healthy Brain**

A hundred and fifty years have elapsed since the original discovery of the microglial cell by Virchow. While this cell type has been well studied, the role of microglia in the pathology of many CNS diseases still remains enigmatic. It is widely accepted that microglial-mediated inflammation contributes to the progression of AD; however, the precise mechanisms through which these cells contribute to AD-related inflammation remains to be elucidated. Microglia are the brain’s tissue macrophage and thus the principal representative of the innate immune system in the central nervous system (CNS). It was widely accepted that microglia originated from peripherally-derived mesodermal progenitors, and invaded the CNS during embryonic and fetal development, prior to formation of the blood brain barrier (Morris et al., 1991, Rezaie and Male, 2002). However, a recent study has elegantly shown that microglia are derived embryonically from yolk sac macrophages which populate the brain around E8.0 and give rise to virtually all adult microglia (Ginhoux et al., 2010). They serve as the
first line of defense in the brain and act to protect the CNS from injury and invading pathogens. They also play an important role in normal tissue maintenance by taking up and disposing cellular debris in the brain paranchyma.

In the mature CNS, microglia only account for 5% of the total glial population in the cerebral cortex (Lawson et al., 1990, Dheen et al., 2007). Despite their small number they play fundamental roles in maintaining brain homeostasis. In the healthy adult brain, microglia have a low turnover rate and comprise a stable population of cells (Lawson et al., 1992). Microglia are evenly dispersed at a density of approximately 6 microglia per cubic millimeter (Davalos et al., 2005). These cells maintain a ramified morphology and express a variety of cell surface markers typical of cells of this lineage, including CD11b, the lectin binding molecule F4/80, the calcium binding protein, Iba1 and MHC II (Perry et al., 1985, Ito et al., 1998).

Originally it was thought that the CNS was populated by a functionally homogeneous population of microglia. However, recent studies have shown that microglia in the brain represent a more phenotypically diverse group of cells that readily react to cues from the extracellular environment by altering their activation status and their expression of phenotypic markers. These cells, like other tissue macrophages, respond to their local environment (Gordon, 2003). Thus, a microglial cell located in one area of the brain may be distinct from that in another, owing to differences in the surrounding environment and the exposure to a different range of extracellular cues.

In recent years, ideas regarding the role of microglia in the healthy and diseased human brain have evolved dramatically. Conventionally, it was thought that microglia in normal healthy brain were “quiescent” and “resting,” however, it is now recognized that
these cells are highly active and very dynamic. The emergence of two-photon microscopy has allowed for a more intimate view of living microglia in their native environment of the brain. Nimmerjahn et al. have described microglial dynamics in a healthy brain (Nimmerjahn et al., 2005). They utilized a transgenic mouse model expressing green fluorescent protein (GFP) from the fractalkine locus and visualized microglial activity in vivo. They found that microglial cells continuously sample the extracellular environment by extending and retracting their processes with limited movement of their cell bodies. Furthermore, during this scanning process, microglia were shown to make repeated contact with astrocytes, neurons and the cerebral vasculature (Davalos et al., 2005). It was estimated that microglia survey the entire brain once every few hours (Nimmerjahn et al., 2005). Additionally, it was shown that these cells were capable of readily detecting CNS injury and quickly migrating to sites of damage within the brain. In a study by Davalos et al., microglia were shown to extend their processes to sites of laser-induced damage within a minute after injury. Following injury, neighboring microglial cells then migrated to the damaged site within 30 minutes of the insult and retracted processes that were not oriented towards the site of damage. It was concluded that astrocytic release of ATP and P2Y12 receptors were necessary for the chemotactic migration of microglial cells to the injury site (Davalos et al., 2005, Haynes et al., 2006). Significantly, resting microglia were also shown to make direct contact with neuronal synapses. These contacts were short lived, lasting no more than 5 minutes, were activity dependent and occurred on an hourly basis. After ischemic injury, microglia made more frequent and prolonged contact with these neuronal synapses (lasting approximately 1 hour) and resulted in the disappearance of presynaptic boutons.
(Wake et al., 2009). These studies taken together, suggest that microglia play a rather active and dynamic role in maintaining homeostasis in a healthy brain and can detect and respond efficiently to CNS injury.

Plaque-Associated Microglial Dynamics in Alzheimer’s disease

In the AD brain most reactive microglia are associated with dense-core plaques, however a few are found in the vicinity of diffuse Aβ deposits (Akiyama and McGeer, 1990, Mackenzie et al., 1995, Akiyama et al., 1999, Stalder et al., 1999). Additionally, the number of microglia and their size directly increases in proportion to the dimensions of the plaque (Wegiel et al., 2001, Sasaki et al., 2002, Wegiel et al., 2003). Microglia in the immediate vicinity of plaques have also been shown to proliferate, allowing for the accumulation of these cells at the periphery of amyloid deposits (Bornemann et al., 2001, Stalder et al., 2001). Plaque-associated microglia have been shown to extend their processes and encompass Aβ deposits and through this association can regulate plaque dynamics in transgenic AD mouse models (Perlmutter et al., 1990, Wisniewski et al., 1992). This association of microglia with amyloid plaques in the brain is consistent in both transgenic animal models of the disease as well as human cases of AD (Itagaki et al., 1989, Frautschy et al., 1998).

A recent study by Yan et al. visualized plaque growth in APP/PS1 AD mouse model using serial in vivo multiphoton microscopy (Yan et al., 2009). Using this method they were able to visualize the growth of individual plaques over a period of time. They demonstrated that plaque growth was more extensive in younger transgenic animals when compared to older ones. Additionally, they found that smaller plaques grew at a greater
rate in comparison to larger plaques. The authors also utilized an inhibitor of γ secretase, and found that treatment of transgenic animals with this compound halted the growth of pre-existing plaques, inhibited the appearance of new plaques and decreased extracellular levels of Aβ by 20-25% (Yan et al., 2009).

Using two-photon microscopy Meyer-Luehmann et al. were able to identify and follow newly formed Aβ plaques in repeated imaging sessions (Meyer-Luehmann et al., 2008). They reported that senile plaque formation was a rapid process, and could identify newly formed plaques within 24 hours of an imaging session, a finding which differs from those of Yan et al. (Yan et al., 2009) Microglia were recruited to these plaques within 1-2 days of their appearance. Despite the recruitment of microglial cells to the newly formed plaque, clearance of the amyloid deposits was not observed. Additionally, it was shown that dystrophic neurites, displaying both dendritic and axonal abnormalities, appeared around the newly formed plaque area and coincided with microglial recruitment (Meyer-Luehmann et al., 2008). However, the contribution of microglia to the neuritic dystrophy was not addressed. Using a similar technique, Bolmont et al., followed single plaques over a period of time, and demonstrated that microglia migrate to plaques while maintaining a highly dynamic interface between the plaques and glial cells. They documented that the migration of microglia to plaques occurred at a rate of about 3 microglia per plaque per month. In contrast to the Meyer-Luehmann study, Bolmont et al. were able to observe the internalization and delivery of Aβ fragments to the lysosomes of microglial cells (Bolmont et al., 2008). These newly developed techniques have proven to be critical our understanding of microglial behavior in the normal as well as diseased brain.
While the development of in vivo two-photon microscopy has illuminated our understanding of microglial activity in the mouse, recent advances in PET based technologies have now allowed for imaging of microglial activation in the living human brain as well. This is achieved by utilizing a carbon 11-labeled compound that recognize the mitochondrial protein TP-18 (also known as the peripheral benzodiazepine receptor) which is expressed at high levels by phenotypically activated microglial cells (Cagnin et al., 2001, Maeda et al., 2004). It also expressed at lower levels in astrocytes (Mankowski et al., 2003, Ji et al., 2008). These studies have helped confirm that, in accordance with the literature, microglial phenotype changes in the diseased brain. Edison et al. were able to determine that the number of activated microglia in the cortex of AD patients was significantly correlated with cognitive decline as assessed using the mini mental status exam scores, rather than amyloid deposition (Cagnin et al., 2001, Versijpt et al., 2003, Edison et al., 2008). However, a study by Wiley et al. yielded contradictory results (Wiley et al., 2009). The increase in number of activated microglial cells in aged AD mouse models has also been validated using this PET-based technique (Maeda et al., 2004, Venneti et al., 2009). Currently, studies using more sensitive TP-18 binding compounds are underway (D'Mello et al., 2009). These studies support the idea that microglial cells are composed of a heterogeneous population that maintains the capacity to shift activation states in response to disease progression.

**Microglia and Inflammation in Alzheimer’s Disease**

There is an extensive literature that documents that inflammation plays an integral role in progression of AD, facilitating Aβ deposition, neuronal loss and cognitive deficits (Patel et al., 2005). The appearance of amyloid plaques in the brain coincides with a
dramatic phenotypic activation of the surrounding microglia which display increased immunoreactivity for CD11b, CD68, complement receptor 3 (CR3) and CD45 (Herber et al., 2007). Postmortem brains from AD patients, as well as brains from APP transgenic animals, display increased levels of inflammatory cytokines as well as chemokines including Interferon γ (INFγ) and Tumor necrosis factor α (TNFα), Interleukin 1β (IL-1β), and Interleukin 6 (IL-6) (Akiyama et al., 2000, Hoozemans et al., 2006, Wyss-Coray, 2006, Heneka and O'Banion, 2007, Rojo et al., 2008). INFγ and TNFα have not only been shown to have toxic effects on neurons but have also been found to reduce levels of insulin degrading enzyme (IDE), a key Aβ degrading protease. This may be a secondary mechanism through which inflammation could increase amyloid deposition. Furthermore, both TNFα and INFγ have been shown to increase the production of Aβ from APP expressing cortical neurons as well as impair the ability of microglial cells to degrade Aβ [45].

The association of reactive microglia with amyloid deposits has been demonstrated in vivo and their ability to secrete a variety of pro-inflammatory cytokines, chemokines, reactive nitrogen and oxygen species as well as acute phase proteins in response to the fibrillar Aβ has been shown in vitro [37-40]. There is an extensive literature that documents the Aβ-induced production of a host of proinflammatory molecules and these findings have been recently reviewed (Akiyama et al., 2000, Halle et al., 2008, Lucin and Wyss-Coray, 2009). Notably, microglial exposure to fibrillary forms of Aβ in vitro provoke the synthesis and secretion of pro-inflammatory cytokines, including IL-1β, IL-6, TNFα, TGF-β, a peptide growth factor and chemokines, MIP-1α, MIP-1β, MIP-2, and most prominently CCL2/MCP1 (Akiyama et al., 2000, Smits et al.,
Aβ-stimulated microglia also produce reactive oxygen species (ROS) and reactive nitrogen species. These cells also elaborate a number of other immune mediators including macrophage colony stimulating factor (M-CSF) and the complement protein, C1q (Lue et al., 1996).

The generation of mouse models lacking inflammatory signaling elements has provided insight into the role of inflammation in AD pathogenesis and Aβ clearance in vivo. AD transgenic mouse models lacking expression of either the INFγ receptor type 1 (GR) or TNF type 1 receptor (TNFR1) display significant decreases in amyloid deposition as well as microglial activation [45, 46]. Deletion of TNFR1 also alleviated Aβ-associated cognitive deficits (He et al., 2007). However, we cannot conclude that inhibition of cytokine signaling promoted microglial clearance of amyloid beta in the animal models since both these studies documented decreases in BACE1 activity as well. Since the deletion of these receptors is global it is difficult to assess the specific contribution of these receptors in the inflammatory response and clearance of Aβ in microglia (He et al., 2007, Yamamoto et al., 2007).

A study by Shaftel et al. took an alternative approach and examined the effects of sustained inflammatory signaling by overexpression of the pro-inflammatory cytokine IL-1β in astrocytes. To the surprise of the authors, sustained IL-1β activation, which was expected to have detrimental effects, resulted in a significant decrease in amyloid deposition in the hippocampus of these mice. They found that IL-1β overexpression led to an increase in the number of activated plaque-associated microglia that were heterogeneous in their phenotype and were positive for either Iba1 or both Iba1 and MHCII and this phenotypic diversity arose from the IL-1β-stimulated recruitment of
blood borne leukocytes into the brain (Shaftel et al., 2007). Similarly, another study conducted by Chakrabarty et al. in which the pro-inflammatory cytokine IL-6 was overexpressed in the brain of murine models of AD using an adenoviral vector, found that amyloid deposition was attenuated in these animals. IL-6 expression resulted in a dramatic increase in gliogenesis in these animals that was associated with enhanced microglial phagocytosis of Aβ, without affecting APP expression or processing (Chakrabarty et al., 2009). From these studies we can conclude that while inflammation has been shown to be detrimental to the brain, some components of this system may be involved in amelioration of AD-related pathology.

*Intrinsic mechanisms that regulate microglial activation in vivo*

Though inflammation plays beneficial roles in pathogen removal it can also be detrimental to the surrounding tissue and result in bystander damage if the response is not downregulated. Thus, the CNS possesses intrinsic mechanisms to suppress microglial activation through cell-cell interactions between microglia and neurons as well as microglia and other glial cell types. One such example is the fractalkine receptor (CX3CR1) expressed on microglial cell surface. The ligand of this receptor, fractalkine (CX3CL1), is expressed on neurons and astrocytes. Interaction of the ligand with the receptor during normal microglial surveillance in the brain prevents microglial activation. It is thought that during neurodegeneration, this ligand-receptor interaction is lost, due to loss of neurons, and contributes to the activation of microglial cells (Ransohoff, 2007). In the Tg2576 mouse model of AD, the number of fractalkine positive cells in the cortex
and hippocampal regions is half that of the wildtype control animals at 9 months of age (Duan et al., 2008).

Similarly, interaction between microglia and neurons via the CD200 receptor located on neurons and CD200 ligand expressed on microglial cells also inhibits microglial-mediated inflammation. Levels of both CD200 and CD200R are reduced in the hippocampus and cortex of AD patients (Walker et al., 2009). It has also been shown in vitro that CD200 interaction with CD200R can attenuate Aβ induced glial activation (Lyons et al., 2007). The microglial transmembrane protein-tyrosine phosphatase CD45 also plays a similar role. Engagement of this receptor with its ligand, CD22, results in the inhibition of the production of pro-inflammatory cytokines by microglia in response to LPS (Mott et al., 2004). CD22 is normally secreted by neurons.

Recent studies have shown that microglial cells can interact via the microglial triggering receptor expressed by myeloid cells 2 (TREM2) with an unidentified neuronal cell surface ligand to suppress microglial-mediated inflammatory cytokine production (Hsieh et al., 2009). AD mouse models express increased levels of TREM2 in plaque-associated microglial cells (Frank et al., 2008). Additionally, two other proteins Signal Regulatory Protein α (SIRPα) expressed on myeloid cells, neurons and astrocytes and CD47 expressed on microglia and neurons can interact and signal bidirectionally to suppress expression of proinflammatory cytokines (Matozaki et al., 2009).

In contrast, the CD40 receptor on microglial cells has been shown to participate in induction of an inflammatory response. Engagement of this receptor with the CD40L leads to the production of TNFα and IL-1β (Tan et al., 1999). Expression of CD40, as well as its ligand CD40L, is increased around Aβ plaque deposits in the AD brain (Togo
et al., 2000, Calingasan et al., 2002). Tg2576 animals deficient for CD40 ligand show a marked reduction in Aβ burden, microglial activation and astrogliosis (Tan et al., 2002). Thus, while there are many intrinsic mechanisms are employed to make sure microglial activation is under tight control, many of these signaling components are compromised in the AD brain.

**Phagocytosis of Aβ by Microglial Cells**

One of the enigmatic features of AD is that while microglia are competent phagocytes, they are unable to efficiently clear amyloid deposits within the brain. Microglial cells have been shown to clear soluble forms of the Aβ peptide by macropinocytosis and are capable of taking up fibrillar Aβ (fAβ) peptides through phagocytosis (Koenigsknecht and Landreth, 2004, Mandrekar et al., 2009). Fibrillar forms of Aβ are recognized by a receptor complex, described in detail below, located on the microglial cell surface. Activation of this complex initiates an intracellular signaling cascade that engages the phagocytic machinery of the cell and results in the phagocytosis of fAβ. Once internalized both soluble and fibrillar species of Aβ are delivered to the endolysosomal system (Paresce et al., 1997b, Bamberger et al., 2003, Bolmont et al., 2008).

While microglial cells possess the ability to take up fibrillar forms of Aβ its seems as though in the AD brain the microglia have a difficult time degrading these dense aggregates and suggests that disease progression may be paralleled by defective microglial clearance mechanisms. Paresce et al. have shown *in vitro*, that murine microglial cells are capable of phagocytosing Aβ, however, they have observed, that once
internalized the peptide can remain undegraded for over 72 hours (Paresce et al., 1997b). In another study it was shown that once microglia internalized Aβ they resecreted the undegraded peptide into the culture medium (Chung et al., 1999).

The conclusion that endogenous microglia are inefficient or unable to effect plaque clearance has been supported by a recent and provocative study by Grathwohl and colleagues (Grathwohl et al., 2009). These authors reported that they could selectively ablate endogenous microglia using a CD11b-driven thymidine kinase gene coupled with ganciclovir administration to the brain of murine models of AD. They found that loss of microglia had no effect on plaque number or size over a 2-4 week period. However, the interpretation of these experiments is confounded by the morbidity and mortality that accompany ganciclovir treatment, the loss of other cell populations besides microglia, most notably pericytes, the loss of the integrity of the vasculature and perturbation of normal homeostatic mechanisms in the brain. While this study was argued to undermine a role for microglia in AD pathogenesis, an alternate interpretation of these data is that it just reinforces the view that endogenous microglia do not normally act to efficiently remodel or clear amyloid deposits in the AD brain.

Many in vitro studies have supported the idea that an inflammatory environment negatively affects the capacity of microglial cells to engage in phagocytosis and clear fibrillar Aβ deposits (Koenigsknecht-Talboo and Landreth, 2005, Zelcer et al., 2007). Yamamoto et al., were able to show in vitro that wildtype microglia were able to take up and degrade Aβ, however, they lost their capacity to degrade the peptide following inflammatory cytokine stimulation, resulting in the retention of Aβ40 and Aβ42 within the cells (Yamamoto et al., 2008). Microglial cells isolated from APP/GRKO animals
(where INFγ signaling is disrupted), were able to take up Aβ and did not lose their
capacity to degrade exogenously supplied Aβ in response to cytokine stimulation
(Yamamoto et al., 2007).

Koenigsknecht-Talboo et al. have shown that inflammatory cytokine treatment
inhibits the ability of microglial cells to phagocytose Aβ that occurs upon engagement of
the fAβ receptor complex, however this treatment did not alter Fc-mediated phagocytosis
by microglia. Indeed, in Aβ vaccination models, microglia can be stimulated to remove
plaque through the activation of the FcR in the presence of an inflammatory environment
(Wisniewski and Konietzko, 2008). Treatment of microglial cells with ibuprofen, known
for its anti-inflammatory actions, rescues impairments in fAβ-induced phagocytosis by
microglia in response to a pro-inflammatory environment (Koenigsknecht-Talboo and
Landreth, 2005). Treatment of microglial cells with anti-inflammatory agents such as
NSAIDs, LXR agonists, PPARγ agonists and anti-inflammatory cytokines can reverse
any phagocytic deficits incurred in response to inflammation (Combs et al., 2001a,
Koenigsknecht-Talboo and Landreth, 2005, Zelcer et al., 2007, Yamamoto et al., 2008).
Similarly, a study conducted by Shie et al examined the effects of the E prostanoid
receptor subtype 2 (EP2) on the ability on microglia to mediate the phagocytosis of Aβ.
Prostaglandin E2 is a product of cyclooxygenases and can bind to a number of E
prostanoid receptors. These authors found that EP2 knockout microglia were more
efficient at clearing Aβ peptides in solution as well as from AD brain sections (Liang et
al., 2005, Shie et al., 2005). Together with the in vitro experiments, these studies suggest
that inhibition of inflammatory signaling reduces microglial activation, enhancing their
ability to mediate Aβ clearance.
In line with these studies, long term use of non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit cyclooxygenases (COXs), have been shown to reduce the risk of AD as well as delay disease progression (McGeer et al., 1996, Vlad et al., 2008). Long term treatment of transgenic AD mouse models with ibuprofen has also been shown to reduce inflammation, ameliorate cognitive deficits and decrease amyloid deposition (Lim et al., 2000, Lim et al., 2001, McKee et al., 2008). These studies taken together suggest that inflammation may impact the ability of microglial cells in the brain to take up and degrade Aβ and therapies geared towards ameliorating the inflammatory response in the AD brain may prove to be a beneficial in treating this disease.

**Microglial Aβ Receptor Complex**

Microglial cells possess a wide variety of cell surface receptors that are capable of recognizing Aβ aggregates and initiating an inflammatory response. Some postulated cell surface Aβ receptors include RAGE, and the formyl peptide receptor-like 1 protein (FPRL1) (Cui et al., 2002, Yamamoto et al., 2007, Halle et al., 2008, Cameron and Landreth, 2009, Salminen et al., 2009). However, the exact role of these receptors in the recognition of fAβ by microglial cells remains controversial.

We have previously shown that fibrillar forms of Aβ can interact with a multicomponent receptor complex on microglial cells. This complex is composed of CD36 (a B-class scavenger receptor), the α6β1 integrin, the integrin associated protein CD47 and scavenger receptor A (Bamberger et al., 2003). Engagement of this receptor complex with fibrillar Aβ leads to the activation of an intracellular signaling cascade, resulting in the phenotypic activation of microglia through NFκB-mediated gene transcription. Additionally, activation of this receptor complex results in increased

Many studies support the idea that the receptor complex plays an important role in AD pathogenesis. Microglia isolated from CD36 null animals, exhibit a decreased levels of cytokine and chemokine secretion as well as reactive oxygen species in response to exposure to fibrillar Aβ species (El Khoury et al., 2003). A study by Hickman et al., described a 2.5 fold increase in levels of IL-1β and TNFα mRNA in a PS1-APP AD mouse model with a parallel two to five fold decrease in mRNA levels of scavenger receptor A (SRA), CD36 and RAGE in comparison to their age matched wildtype littermates. The fall in receptor levels was postulated to be due to the increase in levels of proinflammatory cytokines, as incubation of N9 microglial cells with TNFα decreased the expression of SRA and CD36 and reduced Aβ uptake by cultured microglial cells (Hickman et al., 2008).

Additionally, we have recently reported that the innate immune toll-like receptors (TLRs), and the coreceptor CD14, function as integral members of this fAβ receptor complex. We show that stimulation of both TLR2 and TLR4 by fibrillar Aβ is required for microglial activation (Reed-Geaghan et al., 2009). TLR2, TLR4 and its coreceptor CD14 are necessary to mediate and activate intracellular signaling in response to fibrillar Aβ. Cells deficient in any of these proteins fail to initiate Src-Vav-Rac signaling, resulting in reduced levels of reactive oxygen species and displayed impaired phagocytosis in response to fAβ stimulation. They also failed to induce phosphorylation of IκBα after fAβ stimulation. These data provide strong evidence that TLR2, TLR4 and CD14 function as additional members of the microglial fAβ receptor complex we have
previously described (Reed-Geaghan et al., 2009). These studies demonstrate the recognition of Aβ at the cell surface occurs through the association of immune receptors into a multi-subunit receptor complex that is critical in engaging the phagocytic machinery of the microglial cell as well as inducing the classical activation of microglial cells.

The role of Toll-Like Receptors in inflammation and Aβ clearance

The innate immune system plays a key role in the discrimination between self and non-self. Thus, the host defense system has evolved strategies to detect tissue damage and invading viral and microbial pathogens. Microglial cells are capable of initiating an immune response through the engagement of various pattern recognitions receptors (PRRs) expressed on their cell surface, including the toll-like receptors (TLRs). TLRs are specialized to recognize pathogen associated molecular patterns (PAMPs) that are conserved within a class of microbes. TLRs can also recognize danger associated molecular patterns (DAMPs) which are released after tissue damage. The TLRs are comprised of a family of 13 structurally similar receptors in mammals and are expressed on a variety of immune cells, including B cells, T cells, macrophages, monocytes and dendritic cells. Recognition of PAMPs and DAMPs by TLRs initiates signaling cascades resulting in the production of inflammatory cytokines, proteases and reactive oxygen and nitrogen species leads to the rapid and efficient clearance of these agents and the classical activation of microglia (Kawai and Akira, 2007).

In the brain, TLRs are expressed mainly on microglia, however, recently it has been reported that a subset of TLRs are expressed on neurons and astrocytes (Bsibsi et al., 2002, Jack et al., 2005). Microglia associated with Aβ deposits in AD brains as well
as murine models of AD express elevated levels of TLR 2/4/5/7/9 mRNAs (Frank et al., 2009). Similarly, the AD brain, as well as the brains transgenic murine models of AD, exhibit increased protein levels of CD14, TLR2 and TLR4 (Fassbender et al., 2004, Liu et al., 2005). TLR4, TLR2 and the coreceptor CD14, are of particular importance to Alzheimer’s disease due to their ability to recognize Aβ peptides and oligomers (Fassbender et al., 2004).

Both TLR2 and TLR4 activation have been linked to inflammation in the AD brain. Treatment of microglia with function blocking antibodies to TLR2 and TLR4 results in decreased production of the inflammatory cytokines, IL-6, TNFα and reactive nitrogen species in response to fAβ stimulation. Neutralizing antibodies or genetic deficiencies in CD14 elicit a similar response (Fassbender et al., 2004). Additionally, Walter et al. have shown that media from Aβ-treated wildtype microglia have neurotoxic effects on neurons, while media from Aβ primed TLR4 or CD14 deficient microglia is no longer toxic, suggesting that these receptors are necessary to mediate microglial neurotoxicity (Walter et al., 2007). These data strongly suggest that in microglial cells the TLRs are involved in mediating an inflammatory response to Aβ.

The TLRs as well as the coreceptor CD14 have also been implicated in the uptake of Aβ by microglia (Liu et al., 2005). Deletion of CD14 results in decreased Aβ uptake by microglial cells in comparison to wildtype cells. Tahara et al. have shown that activation of microglial TLR2 and 4 receptors results in increased Aβ phagocytosis (Tahara et al., 2006). Moreover, both TLR2 and TLR4 are necessary for fAβ stimulated phagocytosis(Reed-Geaghan et al., 2009). These in vitro studies strongly implicate TLR2, TLR4 and CD14 in the phagocytosis of Aβ by microglial cells.
While a number of *in vitro* studies have shown that TLR activation promotes inflammation and may play a role in plaque clearance, *in vivo* studies examining the effect of genetic inactivation of the TLRs are simply confusing. APPswe/PSEN1ΔE9 mice (14 month old) expressing an inactive, mutated TLR4, displayed increased levels of both diffuse and dense core-amyloid plaques in both the hippocampus and cortex (Tahara et al., 2006). The authors suggest that TLR4 functions in microglia to mediate the clearance of Aβ and thus, the absence of functional TLR4 results in a dramatic change in Aβ load. In another study, peripheral injection of LPS, a TLR4 ligand, promoted microglial activation and ultimately resulted in the clearance of diffuse, but not compact, amyloid deposits (DiCarlo et al., 2001). Studies conducted by Herber et al. demonstrated a similar result (Herber et al., 2007). Together these studies support a role for TLR4 in microglia activation and promotion of plaque removal. However, a study by Sheng et al reported contradictory results; they demonstrated that LPS treatment increased overall Aβ peptide levels (Sheng et al., 2003).

A different result was reported on examination of TLR2 knockout animals crossed with the APPswe/PSEN1dE9 AD mouse model revealed delayed Aβ deposition and enhanced cognitive decline at 3 and 6 months of age. However, at 9 months of age these animals exhibit comparable levels of amyloid deposition to their wildtype littermates (Richard et al., 2008). Thus, the role of TLRs in AD related pathophysiology remains unclear. These differences in Aβ deposition between the TLR2 and TLR4 deficient mouse models may possibly be attributed to the age of analysis.

A recent study has also described the possible role of another toll-like receptor, TLR9, in Aβ clearance. TLR9 is located in endosomal compartments within the cell and
recognizes CpG DNA from viruses and bacteria that have been internalized (Krieg, 2006). Activation of TLR9 by CpG ODNs (unmethylated cytosine-guanosineoligodeoxynucleotides) has been shown to induce phagocytosis in murine microglial cells in vitro (Ribes et al., 2009). The study by Scholtzova et al. stimulated TLR9 by intraperitoneal injection of CpG ODN. These mice displayed a 66% reduction in cortical amyloid burden as well as an 80% reduction in vascular amyloid deposition. Treatment with CpG ODN also rescued behavioral deficits seen in the radial arm maze. Since CpG ODNs are unable to penetrate the blood brain barrier, it is speculated that the effects of TLR9 activation were on peripheral macrophages which then infiltrated the brain (Scholtzova et al., 2009).

**Alternative Activation of Microglial Cells**

Traditionally, the microglial cell was classified into two primary phenotypic states; ‘quiescent’ or ‘activated’. The transformation of ‘quiescent’ microglia to an ‘activated’ phenotype was associated with inflammation and disease. It is now clear that the phenotypic and functional heterogeneity of microglial cells in the CNS is far more prevalent than previously appreciated and it is slowly being recognized that these cells are highly plastic and play diverse roles in the brain. Several recent studies have highlighted the inherent diversity of microglia and have discovered that they are able to conform into not just two but a variety of different activation states. The past decades of research in AD have focused on the classical, pro-inflammatory activation of microglia. While classical activation is an important host-defense mechanism it is important to down regulate this response and initiate tissue repair to attain homeostasis in the CNS after
insult or injury (Gordon, 2003, Mantovani et al., 2004). Recent studies suggest that this may be achieved by modulating the activation states of local or infiltrating microglial cells. This appreciation of the phenotypic heterogeneity of microglia has had its basis in studies of peripheral macrophage biology. Gordon and colleagues have proposed a classification system that describes the ‘classical’ proinflammatory activation state as M1 and ‘alternative’ activation states as M2 (Martinez et al., 2009). More recent attempts at defining the diverse macrophage phenotypes have employed other nomenclatures (Mosser and Edwards, 2008) (Town et al., 2005) in attempt to reflect the plasticity and heterogeneity of these cells. Indeed, it is now widely appreciated that there is a spectrum of activation states that defy easy classification and that the characteristics of the individual cells is a function of local environmental influences and the capacity of these cells to initiate and resolve the tissue response to pathogens or injury. It is not clear whether these phenotypic distinctions are appropriate for describing the brain resident populations of myeloid lineage cells (Morgan et al., 2005, Martinez et al., 2009).

Classical activation (also referred to as M1 activation) of microglial cells results in the production of Th1-proinflammatory cytokines, NOS2 expression and is characterized by the ability of microglia to present antigen (Mantovani et al., 2004). In peripheral organs, tissue macrophages can acquire non-classical, or ‘alternative’ activation phenotypes. This has been postulated to be an evolutionary adaptation to parasitic infections in which the immune system is unable to remove the activating pathogen. An analogous situation may exist in the brain owing to the inability of microglia to remove Aβ plaques. Alternative activation (or M2 activation) of microglia is typified by the expression of Th2 anti-inflammatory cytokines including IL-10, TGF-β,
IL-4 and IL-13 and the suppression in the expression of Th1-proinflammatory cytokines and NOS2. Alternatively activated microglia have typically been thought to play a role in tissue repair and express genes such as YM1, YM2, RELMα (FIZZ1), CD206 (mannose receptor) and arginase 1 (Arg1) (Colton et al., 2006).

It is important to note, however, that M1 and M2 activation states represent only two different phenotypes in a continuum of macrophage activation which may also encompass “mixed” microglial phenotypes as well (Town et al., 2005). The M2 alternative activation state has been further subdivided into three distinct categories, M2a, M2b and M2c. M2a macrophages are induced by IL-4 and IL-13 and exhibit an anti-inflammatory phenotype. M2b macrophages are unique in that they express high levels of proinflammatory cytokines, characteristic of M1 activation, but also express high levels of the anti-inflammatory cytokine IL-10 (Mosser, 2003). The M2b phenotype is induced by exposure to immune complexes, agonists for IL-1R and TLRs. M2c macrophages are considered to be in an “acquired deactivation” state induced by IL-10, TGFβ, glucocorticoids or contact with apoptotic cells, and are associated with the suppression of the innate immune response. Macrophages exhibiting the M2c phenotype play a role in the phagocytosis of cellular debris and apoptotic cells without the induction of a classical immune response allowing for normal tissue maintenance and repair (Mantovani et al., 2004).

While the M2a, M2b and M2c macrophage activation phenotype has been classified in the periphery, it is unclear whether these states occur in microglia in the brain. Expression of alternative markers have been found in the brain, but it remains to be determined whether microglial population can shift between activation phenotypes from
classical to alternative activation or vice versa, although this seems probable. It is also unclear to what extent both microglial subpopulations coexist in the brain and their distribution in relation to the plaque pathology. In vitro studies have revealed that cultured monocyte-derived macrophages that were polarized to a classical activation state were able to switch their activation status in response to IL-4 and IL-10 treatment (Gratchev et al., 2006).

Recent studies have shown that AD mouse models display a switch in microglial activation status in response to disease progression. A study conducted by Colton et al. demonstrated that two mouse models of AD (Tg2576 and Tg-SwDI) expressed increased levels of mRNA for the alternative activation markers Arg1, mannose receptor and YM1 in comparison to wildtype controls. Brain samples obtained from AD patients showed a similar increase in levels of M2 markers (Colton et al., 2006). Transgenic hAPP mice deficient for C3 also exhibited an alternative activation phenotype, displaying reduced NOS2 production and an increase in levels of IL-4 and IL-10 (Maier et al., 2008). However, a contradictory study conducted by Jimenez et al., describes an age-dependent switch in microglial activation in the hippocampus of the APP/PS1 mouse model. They provide evidence that microglial cells exhibit an M2 alternative activation phenotype early in disease progression which then switches to a classical phenotype in older transgenic mice. Interestingly, the authors found in young APP/PS1 mice, microglial cells surrounding amyloid plaques displayed an alternative activation state and expressed IL-1β and YM1. At later times, following plaque deposition, the microglia alter their activation status and display an increase in TNFα production while down-regulating expression of IL-4 and YM1 (Jimenez et al., 2008). Furthermore, the authors went on to
show that the soluble brain extracts containing small oligomeric species of Aβ from 18 month old APP/PS1 mice, stimulated the production of TNFα by astroglial cells. This suggests that recognition of oligomeric Aβ by microglial cells is a key component in programming these cells towards M1 activation. A recent study conducted by Kiyota et al, induced the expression of the M2 cytokine IL-4 using an adeno-associated viral vector in APP+PS1 mice. CNS expression of IL-4 in an AD mouse model, attenuated amyloid pathology, reduced astro/microgliosis and rescued spatial learning in the radial arm water maze (Kiyota et al., 2010). These data suggest that polarizing the AD afflicted brain milieu to a M2 state may exert beneficial effects on AD pathogenesis. The idea that microglial cells exhibit an age and disease-associated change in activation state is of importance, and the significance of this phenotypic change in ameliorating disease pathology needs to be studied in more depth.

Recently, it has been shown that activation of a nuclear receptor, peroxisome proliferator-activated receptor gamma (PPARγ) controls the activation status of macrophages, promoting acquisition of an M2 phenotype. Similarly, PPARγ and PPARδ activation resulted in induction of M2 activation marker Arg1 and the pro-inflammatory cytokine IL-4 (Gallardo-Soler et al., 2008, Loane et al., 2009). This induction was lost in macrophages from PPARγ and PPARδ knockout mice resulting in results in elevated brain levels of IL-4 and Arg1. However, this study was carried out in peripheral monocytes and it is unknown if PPARγ agonists can induce M2 state in microglial cells within the brain. Since microglia play such an integral role in AD pathophysiology, understanding role of microglial activation states and their subsequent effect on AD
pathology is of importance, for not only understanding disease mechanisms, but also for developing new therapies.

**Infiltration of Peripheral Monocytes into the Brain**

In the periphery, injury results in the recruitment of circulating monocytes to the site of injury, their infiltration into the tissue and their differentiation into macrophages. These cells then phagocytose debris and dying cells as well as secrete pro-inflammatory cytokines and other immune effector molecules. These cells are attracted to the site of injury by the local release of chemokines and cytokines, most prominently CCL2. Infiltrating macrophages then exit the tissue and are removed through the lymphatic system as well as the peripheral circulation, allowing the site of insult to return to pre-injury homeostasis. While this mechanism is useful to remove inflammatory macrophages in the injured tissue, the blood brain barrier (BBB), separating the CNS from the rest of the body, poses as a problem for circulating monocytes to enter and exit the brain (van Rossum et al., 2008).

It is widely accepted that in the CNS microglial cells have a low turnover rate and are capable of self renewal (Chan et al., 2007). However, in AD, the appearance of amyloid plaques is associated with a parallel increase in the number of microglia and astrocytes (Barron, 1995). This had previously been believed to be the result of migration of endogenous microglia and their proliferation in situ. Recently, the question has been reexamined following several reports that peripheral monocytes can traffic into the brain. Moreover, there is evidence that suggests that infiltrating bone marrow-derived microglia
may be more competent and efficient at restricting amyloid plaques in comparison to their endogenous counterparts (Malm et al., 2005, Simard et al., 2006).

A number of recent studies have challenged the idea that the BBB is impermeable and have suggested that infiltrating monocytes could invade the CNS and ameliorate AD related pathology. A study by Simard et al. reported that bone marrow derived monocytes were able to transverse the blood brain barrier from the periphery, invade the brain parenchyma and were able to associate with and clear Aβ deposits. Specifically, they reported that 1% of the brain microglial population was derived from peripherally recruited monocytes (CD11b+Iba1+ cells). These infiltrating cells were found to be associated with about 20% of the β-amyloid plaques found in the AD brain and were able to internalize Aβ deposits (Stalder et al., 2005, Simard et al., 2006, Bolmont et al., 2008). This latter observation is of importance because resident CNS microglia were unable to mediate the clearance of Aβ (Simard et al., 2006). However, two studies published in 2007, challenged this idea when they demonstrated that under physiological conditions, peripherally derived monocytes were unable to cross the BBB and infiltrate the brain, suggesting that the infiltration of monocytes into the brain of the AD models was an artifact of the experimental techniques utilized (Ajami et al., 2007, Mildner et al., 2007b).

Recently, a couple of elegant studies have demonstrated that CCL2 (formerly known as monocyte chemotactic protein-1, MCP-1) may play a key role in the attraction and infiltration of peripheral monocytes into the brain. CCL2 is a member of the β chemokine family; it is a ligand for the G-protein coupled receptor, CCR2. CCL2 functions as a chemo-attractant for microglia and monocytes and its expression is elevated in both activated astrocytes as well as cells of monocytic origin (Calvo et al., 1996,
Glabinski et al., 1996). Stimulation of both microglia and astrocytes with Aβ increases expression of CCL2 (Smits et al., 2002, El Khoury et al., 2003). In the AD brain, CCL2 is found associated with senile plaques as well as in activated microglia isolated from APP transgenic mice (Ishizuka et al., 1997, El Khoury et al., 2007). CCR2 has been shown to be involved in the migration of macrophages to sites of axonal injury (Izikson et al., 2000, Babcock et al., 2003). Overexpression of CCL2 in the Tg2576 mouse model of AD resulted in an increased accumulation of microglial cells and diffuse plaques in the mouse brain (Yamamoto et al., 2005). CCL2 overexpression was also shown to accelerate memory deficits in the Tg2576 mouse model and facilitated Aβ oligomer formation in vitro by microglial cells (Kiyota et al., 2009b). Delivery of a dominant negative CCL2 by administration by AAV1/2 hybrid virus to an AD mouse model resulted in a reduction of microgliosis and amyloid deposition (Kiyota et al., 2009a).

A compelling study by El Khoury and colleagues demonstrated that loss of CCR2 in an AD mouse model was associated with a significant reduction in microglial number and increased levels of brain Aβ. This was paralleled with a decrease in levels of neprilysin, a key Aβ protease. In this study the authors distinguished between peripherally derived monocytes/microglia and endogenous microglia by the expression levels of CD45. Endogenous microglial cells exhibit lower levels of CD45 in comparison to infiltrating cells. Tg2576 animals display a 12 fold increase in levels of CD45hi microglia, however, CCR2 knockout animals had wild type levels of CD45 expressing microglia, suggesting that infiltration of peripheral monocytes/microglia is impaired in the knockout animals (El Khoury et al., 2007). Additionally, a study by D’Mello et al. demonstrated that hepatic inflammation resulted in an increase in cerebral CCL2 levels
and well as an increase in circulating CCR2 positive monocytes. They were able to show that hepatic inflammation induced the expression of CCL2 by cerebral microglia prior to monocyte infiltration into the brain and that infiltration of monocytes was dependent on TNFα signaling (D'Mello et al., 2009). These studies provide compelling evidence that this chemokine and its receptor may play an influential role in the infiltration of peripheral monocytes into the brain.

To determine whether monocytes can infiltrate the brain several other studies have looked at manipulating the expression of CD11c-expressing myeloid cells. CD11c is a cell surface integrin and is most notably expressed by antigen presenting dendritic cells. Dendritic cells (DCs) are part of the mononuclear phagocytic system and were initially thought to be distinct from macrophages. However, it was recently reported that CD11c colocalized within a subset of cells that were positive for the microglial markers Iba1, F4/80 and CD11b (Bulloch et al., 2008). It has been suggested that DCs are “immature macrophages”, however this remains controversial (Hume, 2008).

Butovsky et al. utilized diptheria toxin to selectively ablate dendritic-like innate immune cells in a mouse model of AD. This model was genetically engineered to target toxicity towards bone marrow derived cells that are of myeloid origin and express CD11c. Depletion of CD11c+ cells resulted in a significant increase in amyloid deposition in the brain parenchyma (Butovsky et al., 2007). Town and colleagues have provided additional evidence supporting the concept that monocytes can infiltrate the blood brain barrier and traffic into the brain. They demonstrate that TGF-β receptor signaling through Smad2/3 plays an important role in inhibiting the infiltration of peripheral monocytes/microglia into the brain. TGF-β signaling was disrupted in Tg2576
animals by expression of a dominant negative TGF-β receptor in cells of myeloid lineage using the CD11c promoter. Blockade of TGF-β signaling resulted in decreased plaque pathology (both cerebral and vascular), improved behavior, and an increased number of peripheral CD45hi expressing macrophages surrounding amyloid plaques and cerebral vessels (Town et al., 2008). These infiltrating cells display the cell surface markers CD45⁺CD11b⁺Ly-6C⁻ (Geissmann et al., 2003, Town et al., 2008) and correspond to a population of monocytes that home to inflamed tissues (Geissmann et al., 2003).

However, the evaluation of whether peripheral monocytes can enter the CNS remains problematic. The lack of specific markers to distinguish between CNS-resident microglia and peripheral monocytes has made this a difficult question to answer.

Greissmann and colleagues have described two populations of circulating monocytes, a “inflammatory subset” that is short lived and CX3CR1loCCR2⁺GR1⁺ and a “homeostatic subset” that is CX3CR1hiCCR2⁻GR1⁻(Geissmann et al., 2003). It is though that this “inflammatory subset” of monocytes, which express CCR2, are able to infiltrate the diseased brain. Furthermore, a number of current studies utilize CD45hi and CD11c expression as markers that are limited to infiltrating monocytes. While no definitive studies have been carried out to show that this is in fact the case, a general consensus in the field supports the idea that CD45hi microglia in the brain are derived from peripheral monocytes.

Similarly, another study looked at the ability of perivascular macrophages to clear cerebral amyloid angiopathy (CAA) in an AD transgenic mouse model. The authors utilized a liposome-encapsulated clodronate, an intracellular toxin, to deplete perivascular macrophages in a TgCRND8 mouse model of AD and demonstrated a 5 fold increase in
CAA pathology as a result of the removal of perivascular macrophages (Hawkes and McLaurin, 2009).

This avenue of research is important because it has been suggested that infiltrating monocytes, may represent a phenotypically distinct group of microglia, capable of ameliorating disease pathology, while endogenous microglial cells may maybe arrested in a classically activated state promoting disease progression. If this theory is correct, enhancing infiltration of peripheral monocytes may be beneficial in mediating the suppression of AD related inflammation.

**Nuclear Receptors**

Nuclear receptors (NRs) are ligand-activated transcription factors which provide a critical and direct linkage between the environment and the genome. In the past decade, drugs targeting the nuclear receptors, Peroxisome proliferator-activated receptor γ (PPARγ) and Liver X receptor α (LXRα) have shown to ameliorate pathogenesis in animal models of AD. These type II NRs form obligate heterodimers with the Retinoid X receptors (RXRs) to form a fundamental transcription factor. While the role of these receptors has been well characterized in the periphery, their actions in the brain have only recently been investigated. Additionally, they have been shown to promote the degradation of the Aβ peptides in the brain by activating genes responsible for reverse cholesterol transport (Jiang et al., 2008). Activation of these receptors has been shown to suppress microglial-mediated inflammatory responses both *in vitro* and *in vivo* (Cameron and Landreth, 2010, Mandrekar-Colucci and Landreth, 2010). Recent findings now suggest these receptors may play a key role in microglial activation by acting in concert to dampen the inflammatory response and promote tissue repair (Hong and Tontonoz, 2008). Agonists
of RXRs are postulated to have similar effects on AD through their association and
activation of both PPARγ and LXRα signaling pathways.

**Gene Activation by Nuclear Receptors**

Nuclear receptors are ligand-activated transcription factors that play diverse roles in a variety of signaling pathways involved in development and metabolism. All members of the nuclear receptor family are structurally conserved and act to directly regulate gene expression through their association with sequence-specific elements within the promoter regions of their target genes. Type II nuclear receptors are comprised of three main domains, the amino (N)-terminal activation domain known as the activation function 1 domain (AF1), which is necessary for coactivator recruitment, the carboxy (C)-terminal ligand-binding domain (LBD) and the DNA-binding domain (DBD). The DBD is highly conserved, mediates binding to specific response elements (LXREs or PPREs) on the promoters of specific target genes (Glass and Saijo, 2010).

Type II NRs, including the PPARs and LXRs, form obligate heterodimers with RXR. These heterodimers are retained in the nucleus regardless of their ligand binding status (Klinge et al., 1997). In the absence of ligand the coreceptor complex is constitutively associated with a nuclear corepressor complex consisting of nuclear receptor co-repressor (NCoR) or the silencing mediator of retinoic acid thyroid hormone (SMRT) and histone deactylases (Figure 3). Upon ligand binding a conformational change results in the release of the corepressor complex and the recruitment of a coactivator complex, comprised of CBP, p300 or others. The coactivator complex has
intrinsic histone acetyltransferase activity allowing chromatin decondensation thus promoting transcription of target genes (Figure 3).

RXRs have been classified as “promiscuous” due to their ability to form heterodimers with a variety of other type II NRs. RXR binding partners have been further characterized as either permissive or non-permissive binding partners. Permissive binding partners such as LXR and PPAR can be activated by ligands specific to either receptor in the heterodimer (Figure 4). Heterodimers formed between RXR and non-permissive binding partners, such as the thyroid hormone receptor, can only be activated by ligands specific to the non-permissive binding partner (Desvergne et al., 2006).

Liver X Receptors (LXRs)

LXR receptors are activated by oxysterols, most prominently hydroxylated forms of cholesterol, and play a critical role in the control of whole body cholesterol homeostasis, as well as exerting potent anti-inflammatory actions (Lehmann et al., 1997). Two isoforms of LXR have been identified, LXRα (NR1H3) and LXRβ (NR1H2). LXRβ is ubiquitously expressed while LXRα is abundantly expressed in macrophages (Calkin and Tontonoz, 2010). Due to their ability to regulate cholesterol metabolism, LXR agonists have emerged as possible therapeutic targets for atherosclerosis. However, their therapeutic utility is compromised owing to their induction of hepatic steatosis. LXR agonists have also been shown to play an important role in the CNS. LXRβ knockout mice display adult onset motor degeneration that is accompanied by axonal dystrophy, astrogliosis, and lipid accumulation (Andersson et al., 2005). Animals lacking both LXRα and β have a variety of abnormalities in the brain including the accumulation of
lipid droplets, loss of neurons, astrocytic proliferation, closure of ventricles and abnormalities in vasculature (Wang et al., 2002).

**Peroxisome proliferator-activated receptors (PPARs)**

PPARs have been shown to play essential roles in energy metabolism, adipocyte differentiation, insulin sensitization and tumor suppression. Their endogenous ligands are dietary lipids and their metabolites (Forman et al., 1997). They act as dominant regulators of lipid metabolism through their ability to transactivate genes encoding enzymes of lipid metabolism, providing a key linkage between diet and the genome. This family is comprised of three isoforms, PPARα, PPARγ and PPARβ/δ. PPARα is known to regulate lipid oxidation and inflammation but is not highly expressed in the brain (Kliewer et al., 1994). PPARγ is involved in lipid storage, insulin sensitivity and energy metabolism and has been shown to promote adipocyte differentiation (Kersten et al., 2000). PPARβ/δ is the most abundant isoform and plays a role in fatty acid oxidation (Barish et al., 2006). The actions of this receptor on insulin sensitivity and energy metabolism in peripheral tissues are well described. The biology of these receptors in the brain is less well understood. Thiazolidinedione (TZD) agonists of PPARγ, such as pioglitazone (Actos™) and rosiglitazone (Avandia™) are FDA approved for the treatment of type II diabetes (Lehmann et al., 1995). The natural ligands of PPARγ are long chain fatty acids, eicosanoids, oxidized lipoproteins and lipids (Lehrke and Lazar, 2005). PPARγ has been most widely studied in the CNS and the effects of its agonists in
the brain have been well documented (Landreth et al., 2008). All PPARγ species have
been shown to exert robust anti-inflammatory actions (Chawla, 2010).

Apolipoprotein E and Alzheimer’s Disease

The ApoE gene is the principal genetic risk factor for sporadic forms of AD. Humans express one of three ApoE alleles; ApoE2, ApoE3 and ApoE4, these isoforms differ only by two amino acids. The ApoE4 allele is most important genetic risk factor for AD, while the ApoE2 allele is thought to be protective. Forty percent of people afflicted with AD possess at least one copy of the ApoE4 allele which increases the risk for AD by 3-4 fold. Possession of two copies of the ApoE4 allele increases the risk factor for AD by 12-fold. The isoform-specific actions of ApoE on amyloid deposition have been demonstrated in APP mice expressing the human ApoE isoforms. Mice expressing the ApoE4 isoform exhibited higher levels of Aβ deposition in comparison to ApoE3 or ApoE2 expressing animals (Fagan et al., 2002). Interestingly, APP-expressing transgenic mice lacking murine apoe failed to develop compact, but not diffuse, plaques suggesting a role for this protein in the deposition of Aβ in the brain. It is of interest to note, however, that while no compact plaques were observed, in the absence of ApoE, the total levels of Aβ within the brains of these animals was significantly elevated (Bales et al., 1999), arguing for a role in Aβ clearance.

ApoE acts to scaffold the formation of high density lipoproteins (HDL) that function to transport cholesterol and lipids throughout the body and in the brain. In the brain, ApoE is primarily synthesized and secreted by astrocytes although it is
expressed at lower levels by microglia. The lipidation of ApoE is carried out primarily by the actions of the ATP-binding cassette transporter A1 (ABCA1) in the brain, which transfers phospholipids and cholesterol to ApoE, forming HDL particles. The size of the HDL particle is proportionate to its lipid content. The lipidation of ApoE governs its functions including its conformation, interactions with membrane receptors and its interaction with Aβ (LaDu et al., 1995, Fisher and Ryan, 1999). ApoE4 is associated with smaller, poorly lipated HDL particles (Jiang et al., 2008). Numerous studies have shown that both PPARγ and LXRα induce the expression of ApoE and ABCA1 and it is through the expression of these proteins that they exert their effects on amyloid pathology.

Nuclear Receptors and Amyloid Clearance in Mouse Models of AD

Genetic studies have long suggested a relationship between dyslipidemia, high cholesterol levels and AD risk. Genes associated with cholesterol regulation such as APOE, ABCA1, LXRβ, ACAT and LRP1 have also been shown to share a linkage with AD (Puglielli et al., 2003, Wollmer et al., 2003). While genes of cholesterol metabolism, and most prominently the APOE4 allele, are known to play a crucial role in AD pathogenesis, the exact mechanisms through which they confer susceptibility to AD is just recently being understood. Jiang et al. was the first to demonstrate lipdated ApoE species act to promote Aβ proteolysis, providing a mechanistic linkage between the major genetic risk factor for AD and the normal clearance of Aβ from the brain. They were able to show that the lipidation of ApoE enhanced the degradation of soluble species of
Aβ by neprilysin in the endolytic compartments of microglia as well as extra-cellularly through the actions of the insulin-degrading enzyme (IDE) (Jiang et al., 2008). Both ABCA1 and ApoE are important mediators of Aβ clearance since microglia lacking apoE or abca1 lost the capacity to degrade soluble Aβ. Importantly, this study utilized the LXR agonist, GW3965, to activate the LXRαs and induce the expression of both ApoE and ABCA1. Significantly, a four month treatment of Tg2576 mice with GW3965 reduced plaque deposition by over 50% and improved contextual memory in these animals (Jiang et al., 2008). Importantly, the ability for microglial cells to degrade Aβ was ApoE isoform-dependent (Jiang et al., 2008). Indeed, macrophages expressing the ApoE2 allele were the most efficient at degrading Aβ, followed by ApoE3, then ApoE4; microglia lacking ApoE were the least efficient at degrading Aβ (Jiang et al., 2008, Zhao et al., 2009). Studies conducted by other laboratories have also shown LXR activation has beneficial effects on amyloid deposition and memory retention in mouse models of AD (Koldamova et al., 2005b, Burns et al., 2006b, Riddell et al., 2007). LXR activation was also shown to suppress amyloid deposition and improve behavior in APP23 mice induced by a high fat diet (Fitz et al., 2010). Consistent with this hypothesis, it was shown by Zelcer et al. that genetic inactivation of LXRα or LXRβ decreased levels of both ApoE and ABCA1 protein levels in APP/PS1 mice exacerbated plaque pathology (Zelcer et al., 2007).

The importance of ABCA1 function in Aβ clearance was confirmed in three independent studies where the abca1 gene was inactivated in four different APP-expressing transgenic mouse models. The loss of abca1 resulted in not only the reduction of ApoE levels, but also a paradoxical increase in Aβ deposition in the brain.
parenchyma of these animals owing to enhanced deposition of poorly lipidated ApoE in the brain (Hirsch-Reinshagen et al., 2005, Koldamova et al., 2005a, Wahrle et al., 2005). Conversely, overexpression of ABCA1 in a mouse model of AD, was shown to decrease both soluble and fibrillar pools of Aβ in 12 month old mice and reduce plaque burden (Wahrle et al., 2008).

The vital role of ABCA1 in LXR-mediated amyloid clearance was demonstrated in a recent study by Donkin et al. In this study, AD transgenic mice that expressed or lacked abca1 were treated with the LXR agonist GW3965. APP/PS1 mice treated with high or low doses of GW3965 showed elevated brain levels of ABCA1 and ApoE, decreased amyloid burden and significant improvement in memory. However, APP/PS1 mice lacking abca1 displayed little change in brain ApoE levels, no reduction in amyloid load or behavioral improvements. These studies, taken together, suggest that LXRs are excellent therapeutic targets for AD and that both ABCA1 and ApoE are necessary for the beneficial effects of LXR agonists (Donkin et al., 2010).

PPARγ was recognized as a therapeutic target for AD about a decade ago, owing not only to its actions on inflammation, but also its effects on insulin sensitization and energy metabolism (Landreth and Heneka, 2001, Heneka and Landreth, 2007, Landreth et al., 2008). Similar to LXRs, PPARγ activation can also induce the expression of both ABCA1 and ApoE. Additionally, PPARγ can also induce the expression of LXRα creating a metabolically linked cycle. This critical feedback loop was first described by Chawla et al. in macrophages and is critical for PPARγ mediated degradation of Aβ (Chawla et al., 2001).
The synthetic thiazolidinedione (TZD) PPAR\(\gamma\) agonists, are widely prescribed for the treatment of type II diabetes mellitus, and have also been shown to be efficacious in a number of CNS disease models (Kersten et al., 2000). Currently, currently two TZD agonists, Actos\(^{TM}\) (pioglitazone) and Avandia\(^{TM}\) (rosiglitazone) are FDA approved for the treatment of diabetes. However, the use of these drugs for CNS-targeted disease treatments is compromised due to their poor blood brain barrier (BBB) penetrance. The permeability of pioglitazone across the BBB is poor, and rosiglitazone is even less permeable and subject to P-glycoprotein-mediated efflux from the brain (Hemauer et al., 2010). Camacho et al first reported a mechanism by which PPARs affected A\(\beta\). They demonstrated that activation or overexpression of PPAR\(\gamma\) let to a dramatic facilitation of A\(\beta\) clearance from the media of neuronal and non-neuronal cells (Camacho et al., 2004). A number of recent studies employing AD mouse models have been carried out and have confirmed the utility of these synthetic agonists in AD disease pathogenesis.

In a study carried out by Yan et al., 12 month old Tg2576 animals were treated orally with 20 mg/kg/day of pioglitazone for 4 months. These animals did not exhibit a change in plaque pathology, however, showed a trend towards a decrease in soluble A\(\beta_{42}\) levels (Yan et al., 2003). When a higher dose of pioglitazone (7days/40mg/kg/day) was employed in 10 month old transgenic mice overexpressing the APP V717I mutation, a 20-25% decrease in plaque burden was observed with significant reduction in A\(\beta_{42}\) levels within the brains of these animals (Heneka et al., 2005). This was the first study that provided conclusive evidence for the utility of PPAR\(\gamma\) agonists in an animal model of AD.
Pederson and colleagues examined the effects of rosiglitazone and found that activation of PPAR\(\gamma\) ameliorated behavioral deficits in the Tg2576 AD mouse model. However, these animals displayed no changes in plaque pathology, but had reduced brain A\(\beta_{42}\) levels. Since the BBB permeability of rosiglitazone is poor the authors postulated that its effects are due to the peripheral actions of this drug and concluded the improvements in behavior were due to a suppression of plasma glucocorticoid levels (Pedersen and Flynn, 2004). A more recent study carried out by Toledo et al. observed the effects of long term rosiglitazone treatments in APP/PS1 animals. These animals were treated with a low dose of rosiglitazone (3mg/kg/day) for 12 weeks and evaluated for plaque deposition and behavior. These animals displayed an approximate 50% decrease in amyloid deposition, a decrease in A\(\beta\) oligomers, preservation of pre- and post-synaptic proteins and the attenuation of cognitive deficits in the Morris water maze. The authors argue that the effects of rosiglitazone were due to the activation of the wnt signaling cascade which they show by an increase in \(\beta\)-catenin expression and a decrease in GSK-3\(\beta\) levels (Toledo and Inestrosa, 2010). In a study by Escribano et al., 9 month old J20 animals were treated with 5mg/kg/day of rosiglitazone for a period of 4 months (Escribano et al., 2010). After four weeks of treatment, the animals showed significant improvements in the object recognition task. After four months of treatment, the 13 month old J20 animals showed a 50% reduction in levels of A\(\beta_{40}\) and A\(\beta_{42}\). The authors also showed a decrease in levels of A\(\beta^{*56}\). While the authors did not detect an increase in ApoE levels in the treated animals they did observe a modest increase in ABCA1 levels and argue that the enhanced A\(\beta\) clearance could be attributed to an increase in lipidation of ApoE by ABCA1 (Escribano et al., 2010). Another study reported a
reversal of associative learning and memory deficits in 9 month old Tg2576 animals after 1 month of rosiglitazone treatments (30mg/kg/day). However, this effect was not observed in 5 or 13 month old Tg2576 animals (Rodriguez-Rivera et al., 2011).

Importantly, PPARγ activation by pioglitazone has also been shown to rescue cerebrovascular function in an aged AD mouse model. A study conducted on 14 month old APP V717I animals treated with 20mg/kg/day of pioglitazone for 6-8 weeks showed that PPARγ activation rescued deficits in neurometabolic coupling and cholinergic denervation. These authors observed no effects of pioglitazone on plaque load in these animals, but this could be attributed to the low drug dose utilized in this study (Nicolakakis et al., 2008).

**Nuclear Receptors and Inflammation**

The appearance of amyloid deposition in the AD brain coincides with a dramatic phenotypic activation of microglial cells in the surrounding area. These cells are stably associated with the plaque and extend their processes deep within the plaque core. Microglial cells are able to detect Aβ through a multi-component cell surface receptor complex (Bamberger et al., 2003). More recently, Toll Like Receptor 2 (TLR2), 4 (TLR4) and 6 (TLR6) and their coreceptors CD14 and CD36 have been shown to participate in this complex (Reed-Geaghan et al., 2009). Activation of this cell surface receptor complex initiates intracellular signaling cascades that leads to NFκB-mediated proinflammatory gene transcription. This results in the production of pro-inflammatory cytokines, chemokines and the production of reactive nitrogen and oxygen species. In
vitro, fibrillar Aβ stimulation stimulates phagocytosis, however, in vivo this effect is not observed and the plaque-associated microglial cells are described to be phagocytically inactive (Koenigsknecht and Landreth, 2004).

The deposition of fibrillar Aβ in the AD brain results in the recruitment of microglia to the plaques owing to their expression of CCL2, which acts to attract microglia (Glabinski et al., 1996). The number of microglial cells surrounding a plaque is proportional to the dimensions of the deposits. This seems to be an ideal situation since microglia are competent phagocytes and are found in abundance surrounding amyloid deposits in the AD brain. It would be assumed that they would clear the deposits and maintain brain Aβ homeostasis. However, this is not the case, the deposition of plaques and the recruitment of microglia to these deposits does not result in their removal but leads to the sustained activation of microglia and chronic production of inflammatory agents. While in normal circumstances inflammation plays a beneficial role in pathogen removal, in AD, the persistence of inflammation is detrimental to the surrounding tissue (Cameron and Landreth, 2010).

There is an extensive literature documenting the effects of chronic inflammation in AD pathogenesis. Notably, studies aimed at alleviating the inflammatory response in AD have been shown to have a beneficial effect on disease progression. Epidemiological studies have also reported that chronic use of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with a dramatic reduction in the incidence of AD (Vlad et al., 2008). Activation of LXR, PPAR and RXR have all been shown to exert robust anti-inflammatory actions (Combs et al., 2001a, Hong and Tontonoz, 2008, Hoi et al., 2010, Nunez et al., 2010). Indeed, treatment of AD mouse models with LXR or PPAR
agonists has resulted in the suppression of microglial activation (Yan et al., 2003, Koldamova et al., 2005b, Riddell et al., 2007, Zelcer et al., 2007, Toledo and Inestrosa, 2010). The anti-inflammatory effects of these receptors are due in part to their functional inactivation of the NFκB promoters.

**Gene Repression Mediated by Nuclear Receptors**

A number of different mechanisms have been proposed to explain how nuclear receptors repress inflammatory genes, including cross-coupling, co-activator squelching and corepressor interference (Li et al., 2000, Subbaramaiah et al., 2001, Pascual et al., 2005). The most compelling of these mechanisms, corepressor interference, was first described by Glass and colleagues in 2005. Normally, inflammatory genes are repressed through the association of the co-repressors NCoR and SMRT acting on kBRE elements. They were able to show that ligand dependent SUMOylation of PPARγ directs the nuclear receptor to NFκB positioned on the promoters of inflammatory genes, thereby stabilizing co-repressor complexes and inhibiting inflammatory gene expression (Pascual et al., 2005). Both PPARs and LXRs have been shown to become SUMOylated after ligand binding (Glass and Saijo, 2010) (Figure 1). The ability of RXRs to be SUMOylated is still unknown. Owing to their ability to broadly inhibit inflammatory gene expression as well as degrade Aβ, LXRs, PPARs and perhaps RXRs serve as an attractive therapeutic target for AD.
Nuclear Receptors and Macrophage/Microglial Activation status

One of the enigmas in AD pathogenesis is the inability of microglial cells to clear amyloid deposits even though they are found associated with these structures and are competent phagocytes. Indeed, immunotherapy with anti-Aβ antibodies demonstrated that these cells retain the capacity to phagocytose and remove amyloid deposits when appropriately stimulated (Schenk et al., 1999). It is now appreciated that microglial cells can exhibit a variety of activation states and this finding may explain sustained microglial activation and the effects they exert in the AD brain.

Although macrophage activation states have been extensively characterized in the periphery it is unknown to what extent microglia can acquire these various states in the brain. A study by Colton et al. demonstrated that AD brains as well as mouse models show a switch in microglial activation status in response to disease progression. In this study it was shown that samples from human AD brains as well as two aged mouse models of AD showed increased mRNA levels of the M2 markers, Arg1 and Ym1, when compared to age matched controls (Colton et al., 2006). It is thought that the environment of the AD brain alters microglial response to fibrillar forms of Aβ and a question of importance now is how microglial polarization not only affects the inflammatory milieu of the brain but how it affects Aβ clearance.

Importantly, In vitro studies have shown that M2 polarization of microglia using M-CSF or IL-4 promote Aβ phagocytosis (Koenigsknecht-Talboo and Landreth, 2005, Majumdar et al., 2008). Systemic administration of M-CSF also cleared amyloid deposition in a mouse model of AD although how this is linked to the acquisition of a M2 status in microglia is unclear (Boissonneault et al., 2009). Activation of LXRs, PPARs
and RXRs, act to polarize microglia to M2 states due to their suppression of NFκB gene induction but also through transactivation of PPARγ genes that induce the M2 states. Recently, it has been shown that activation of PPARγ promotes the acquisition of an M2 phenotype in peripheral macrophages. Activation of PPARγ or PPARδ has been shown to induce Arg1 and IL-4 expression (Gallardo-Soler et al., 2008, Loane et al., 2009).

Indeed, Odegaard et al. discovered a putative PPRE in the distal enhancer of the Arg1 gene (Odegaard et al., 2007). Studies have shown disruption of PPARγ or PPARδ in myeloid cells results in impaired maturation of alternative macrophage activation (Odegaard et al., 2007, Kang et al., 2008, Odegaard et al., 2008). PPARδ and LXR receptors have been shown to also play a role in M2c polarization since ligand activation of these receptors promotes phagocytosis of apoptotic cell bodies (Mukundan et al., 2009, N et al., 2009). Similarly, stimulation of microglia with the LXR agonist, GW3965, acts simultaneously to suppress inflammation and promote fibrillar Aβ stimulated phagocytosis (Zelcer et al., 2007).
Figure 1: APP Processing
Figure 2: Aβ Aggregation and Deposition
Figure 3: In the absence of ligand, type II nuclear receptors heterodimerize with RXR, bind response elements located on the promoters of target genes in association with a corepressor complex actively repressing the expression of target genes (A). In the presence of ligands, the receptor heterodimer undergoes a conformational change, resulting in the exchange of the corepressor complex for a coactivator complex and allowing for gene transcription (B). Upon ligand binding, PPARγ becomes sumoylated and recruited to the NFκB-corepressor complex. This interaction secures the corepressor complex on the κBRE promoter elements, preventing its stimulus-induced dismissal and maintains the NFκB inflammatory genes in a repressed state (C).
A.

B.

C.

Agonist

PPRE/LXRE

Coactivator Complex

NR

RXR

Target Gene Induction

Corepressor Complex

NR

RXR

Sumo

Corepressor Complex

p50

p65

κBRE

Inflammation

TNFα

IL1β

iNOS

Cox2
**Figure 4:** Activation of nuclear receptors PPARγ and LXR induce the expression of genes necessary for insulin sensitization and lipid metabolism. Interestingly, activation of PPARγ induces LXRα and LXRα induces expression of PPARγ, perpetuating a positive-feedback loop (A). RXR activation induces the activation of their permissive heterodimer binding partners, resulting in the expression of both PPARγ and LXRα target genes (B).
Chapter 2
Microglia mediate the clearance of soluble Aβ through fluid phase macropinocytosis

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Abstract
Alzheimer’s disease is characterized by the progressive deposition of beta-amyloid (Aβ) within the brain parenchyma and its subsequent accumulation into senile plaques. Pathogenesis of the disease is associated with perturbations in Aβ homeostasis and the inefficient clearance of these soluble and insoluble peptides from the brain. Microglia have been reported to mediate the clearance of fibrillar Aβ (fAβ) through receptor-mediated phagocytosis; however, their participation in clearance of soluble Aβ peptides (sAβ) is largely unknown. We report that microglia internalize sAβ from the extracellular milieu through a non-saturable, fluid phase macropinocytic mechanism that is distinct from phagocytosis and receptor-mediated endocytosis both in vitro and in vivo. The uptake of sAβ is dependent on both actin and tubulin dynamics and does not involve clathrin assembly, coated vesicles or membrane cholesterol. Upon internalization, fluorescently-labeled sAβ colocalizes to pinocytic vesicles. Microglia rapidly traffic these soluble peptides into late endolysosomal compartments where they are subject to degradation. Additionally, we demonstrate that the uptake of sAβ and fAβ occurs largely through distinct mechanisms and upon internalization are segregated into separate subcellular vesicular compartments. Significantly, we found that upon proteolytic degradation of fluorescently-labeled sAβ the fluorescent chromophore is retained by the microglial cell. These studies identify an important mechanism through which microglial cells participate in the maintenance of Aβ homeostasis, through their capacity to constitutively clear sAβ peptides from the brain.

**Introduction**
The deposition of amyloid beta (Aβ) in the extracellular space of the brain is a pathological hallmark of Alzheimer’s disease (AD). AD pathogenesis is associated with alterations in Aβ homeostasis resulting in an accumulation of Aβ peptides within the brain parenchyma (Tanzi and Bertram, 2005, Wang et al., 2006).

Microglia are the brain’s tissue macrophages and the primary immune effectors within the CNS. These cells are responsible for normal tissue maintenance and continually sample the extracellular environment. They are highly dynamic and respond rapidly to perturbations within the brain. Additionally, they take up solutes and nutrients through endocytosis (Kreutzberg, 1996, Nimmerjahn et al., 2005).

Endocytosis encompasses three primary mechanisms: phagocytosis, receptor-mediated endocytosis and pinocytosis. Phagocytosis involves the uptake of large particles whose internalization is stimulated through its interaction with cell surface receptors, engaging the cell’s phagocytic machinery (Stuart and Ezekowitz, 2005). We have previously shown that fibrillar Aβ (fAβ) interacts with a multicomponent cell surface receptor complex (Bamberger et al., 2003) stimulating its phagocytic uptake (Knauer et al., 1992; Koenigsknecht & Landreth, 2004). Receptor-mediated endocytosis is a mechanistically distinct process, elicited by ligand binding to a receptor on the cell surface, resulting in the internalization of the receptor and its ligand within clathrin-coated or uncoated vesicles (Mellman, 1996, Conner and Schmid, 2003, Kirkham and Parton, 2005, Mills, 2007). Pinocytosis can occur through two separate pathways: micropinocytosis or macropinocytosis, and is typically associated with the uptake of solutes from the extracellular milieu. Micropinocytic vesicles are no larger than 0.1µm in diameter and may be caveolin-coated. Micropinosome formation is independent of actin
and occurs within cholesterol-rich lipid domains of the plasma membrane (Parton and Richards, 2003). In contrast, macropinocytic vesicles are formed by the closure of membrane ruffles, a process dependent on both actin and tubulin (Swanson and Watts, 1995, Conner and Schmid, 2003).

Soluble or small oligomeric forms of Aβ have been postulated to have deleterious actions in the brain and promote disease progression (Lue et al., 1999, Lacor et al., 2007) inducing changes in synaptic function, behavioral deficits and promoting neuronal degeneration (Lue et al., 1999, Kim et al., 2003, Lacor et al., 2007). Additionally, levels of sAβ within human brain tissue has been shown to correlate with disease severity (McLean et al., 1999, Wang et al., 1999). Little is known about the intrinsic cellular mechanisms through which sAβ is cleared from the brain. In the present study we have investigated how microglia take up sAβ. We demonstrate that macropinocytic uptake of sAβ and its subsequent proteolytic degradation represents a significant mechanism mediating Aβ clearance from the extracellular milieu. Although internalization of the soluble peptide is not limited to microglia, these cells are the most efficient in the endocytosis of the peptide in vitro. Significantly, we report that microglia can effectively degrade fluorescently labeled sAβ, but the fluorophores typically used to label these peptides are retained within the cell, demonstrating the limitation of the utility of these preparations.

Methods and Materials
Reagents:

The glutathione S-transferase (GST)-CD36-(93-120) peptide was a gift from Dr. Maria Febrarrio (Cleveland Clinic Foundation, Cleveland, OH). Invasin was a gift from Dr. Ralph Isberg (Tufts University, Medford, MA). Cytochalasin D, fucoidin, nocodazole and filipin were all purchased from Sigma-Aldrich (St. Louis, MO). Cytochalasin D, filipin and nocodazole were reconstituted in DMSO and fucoidin was dissolved in sterile distilled water. The 4N1K peptide was purchased from Bachem (Philadelphia, PA) and reconstituted in sterile distilled water. Lamp1 and Lamp2 antibodies were a kind gift from Dr. Douglas M. Fambrough (Johns Hopkins University, Baltimore, MD). Rap was a gift from Dr. Guojun Bu (Washington University, St. Louis, MO).

Tissue Culture:

The immortalized BV-2 murine microglial cell line was grown and maintained in DMEM containing gentamycin and 2% fetal bovine serum in 5% CO₂ (Blasi et al., 1990).

Primary microglial cells were derived from the brains of C57BL/6 mice at postnatal day 1-2 as previously described (McDonald et al., 1997). Cells were maintained in DMEM/F12 (Gibco, Inc.) containing 1% penicillin/streptomycin and 20% fetal bovine serum (FBS), pH 7.4. Astrocytes were separated from the microglial cultures using a mild trypsinization protocol described by Saura et al. (Saura et al., 2003).

The SHS5Y, neuronal cell line, was maintained in DMEM/F12 containing 1% penicillin/streptomycin and 10% FBS, pH 7.4 in 5% CO₂.

Murine embryonic fibroblasts (MEFs) were isolated from P0 embryos and maintained in DMEM containing 10% FBS and 1% Pen/Strep, pH 7.4 in 5% CO₂.

Aβ Preparation:
Lyophilized Aβ_{1-42} (American Peptide, Inc.) was dissolved to a final concentration of 1 mg/ml in DMSO and stored at -80°C until use. Fluorescently-labeled soluble Aβ_{1-42} was prepared by dissolving the lyophilized peptide to a concentration of 2 mM in 0.1 M sodium bicarbonate for conjugation with Alexa488 or 50 mM sodium borate for conjugation with the Cy3 fluorophore. The Aβ was labeled with Cy3 (Amersham Biosciences) or Alexa488 (Molecular Probes) according to the manufacturer’s protocol. The reaction mixture was allowed to fibrillize at 37°C overnight after which unincorporated dyes were removed by ultracentrifugation at 100,000 x g and the supernatant discarded. The pellet was then resuspended in DMSO, sonicated and subjected to ultracentrifugation at 100,000 x g for 1 hour at 4°C. The supernatant contains the operationally defined “soluble Aβ” which is likely a mixture of primarily monomeric and small oligomeric species and runs with a mobility corresponding to 4 kDa on SDS-PAGE. Sonication and ultracentrifugation was repeated until most of the pellet fraction was solubilized in DMSO. The remaining pellet was dissolved in sterile ddH₂O and comprised the fAβ preparation. Protein concentration was quantified using the BCA method (Pierce, Rockford, IL).

**Immunocytochemistry:**

Murine BV-2 microglial cells and primary murine microglia were plated on coverslips in 24-well plates at a density of 1 x 10⁵ cells/well for 18 hours. The media was removed and replaced with serum free DMEM or DMEM/F12 at the time of treatment. Cells were treated with 2 μg/ml of sCy3-Aβ or sAlexa488-Aβ for 3 hours. Cells were washed three times with cold PBS and fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton. After permeabilization, cells were stained with DAPI (10 minutes). For
co-localization studies, after permeabilization, cells were blocked in 5% normal goat serum for 1 hour. Primary antibodies for Lamp1/Lamp2 were used at 1:50 dilution and anti-Rab5B (Santa Cruz, sc-598) at 1:250 dilution. Cells were incubated with primary antibodies for 1.5 hours and then washed three times with PBS and incubated with secondary antibodies conjugated to Alexa-fluorphores at a 1:100 dilution for 40 minutes. Coverslips were mounted on glass slides and observed using a Zeiss LSM 510 confocal microscope.

_Uptake of 20 nm microspheres or 1.0 µm microspheres._

BV-2 cells were seeded on coverslips in 24-well plates as described above. Fluorescent 1 µm or 20 nm microspheres (Molecular Probes) were blocked with 0.5 mg/ml BSA in 50% PBS solution. Cells were incubated with microspheres or beads for 20 minutes before they were fixed, permeabilized and stained with DAPI (Koenigsknecht and Landreth, 2004). Coverslips were mounted on glass slides and observed using a Zeiss LSM 510 confocal microscope.

_Flow cytometry:_

Murine microglial BV-2 cells and primary murine microglia were plated at a density of 5x10^5 cells/well in a 6-well plate overnight in DMEM containing 2% FBS. The following morning the media was replaced with serum-free DMEM and cells were incubated with 2 µg/ml soluble Alexa488-Aβ for 3 hours. For experiments in which antagonists were utilized, BV-2 cells were incubated with the inhibitor for 30 minutes prior to the addition of soluble Aβ (2 µg/ml). Cells were then washed with cold PBS and fixed with 4% paraformaldehyde. Following fixation, cells were washed with PBS and collected for analysis by flow cytometry using the EPICS-XL.
Live Cell Imaging:

BV-2 microglial cells were plated on Delta T tissue culture plates at a density of 5x10^5 cells/plate. Cells were incubated overnight in DMEM containing 2% FBS. The following day, culture media was replaced with serum-free DMEM and live cell imaging was performed using a Zeiss LSM 510 confocal microscope. Both Cy3-Aβ (2 µg/ml) and Lysotracker (Molecular Probes, Green DND-26) were added to the culture media at the same time. Lysotracker was used according to the manufacturer’s protocol.

Two-Photon Microscopy

The cranial window procedure was performed on 7 month old CX3CR1/GFP+/+ mice (Jung et al. 2000) as described before (D'Amore et al., 2003, Brendza et al., 2005). Briefly, the mice were anesthetized with avertin prior to placement in a stereotaxic device. After removal of the hair and cleaning the skin with 70% isopropanol, the skin was cut away from the eyes to the base of the skull. A circle was carved in the skull approximately 8 mm in diameter that crossed the midline and was just slightly anterior to both bregma and lambda. This portion of the skull was removed and the exposed brain region was irrigated with PBS and packed with gel foam. Gel foam was removed and Cy3 labeled soluble Aβ was placed on the surface of the brain. The exposed brain region was then covered with a piece of coverglass held in place by dental cement and super glue.

A ring of paraffin was placed over the dental cement to create a chamber to hold water for imaging. Approximately 45 minutes after the Cy3 labeled sAβ was placed on the brain the anesthetized mouse was placed on the stage of a 2-photon microscope (Zeiss LSM 510 Meta NLO system with Coherent Chameleon Ti:Sa laser). For simultaneous
imaging of GFP and Cy3 a wavelength of 900 nm was used. Fluorescence emission of GFP and Cy3 was collected in the ranges of 500-550 nm and LP560 nm filter settings respectively.

After *in vivo* imaging the mouse was assessed to confirm deliverance of the sAβ peptide in the brain. Following *in vivo* imaging the brain, the mouse was perfused with PBS containing 0.3% heparin and the brain was removed. The brain was immersion fixed in 4% paraformaldehyde for 24 hours before being placed in 30% sucrose. Brains were sectioned at 20 micron on a cryostat and images were obtained as a z-series stack using the Zeis LSM 510 confocal microscope.

*Western Blotting:*

BV-2 cells were plated in 6 well plates at a density of 5x10^5 cells/well. After 18 hours of incubation in DMEM containing 2% FBS, media was replaced with serum-free DMEM. Cells were then incubated with unlabeled or Alexa488-labeled Aβ for 3 hours. Cells were washed with cold PBS and lysed using RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Protein concentrations of the cell lysates were measured using the BCA method. Western blot analysis of Aβ was carried out on 4-12% Bis-Tris gels (Invitrogen, Carlsbad, California). Aβ was detected using the anti-human Aβ antibody, 6E10 (Covance, Dedham, MA), at a 1:2000 dilution and was detected by enhanced chemiluminescence (Pierce, Rockford, IL). β-tubulin (Santa Cruz, sc-5274) served as a loading control.

*ELISA:*

BV-2 cells were plated overnight in 24 well plates at a density of 1x10^5 cells/well in DMEM containing 2% FBS. Cells were incubated in fresh serum-free DMEM
containing 2 µg/ml of soluble Alexa-488 or Cy3 labeled or unlabeled Aβ peptides (2 µg/ml) for 3 hours. Cells were lysed in 1% SDS-containing protease inhibitor cocktail. Aβ ELISA’s were performed using 6E10 (Covance, Dedham, MA) as the capture antibody and 4G8-HRP (Covance, SIG-39245-200, Dedham, MA) as the detection antibody. Synthetic Aβ_{42} was used to generate a standard curve. Plates were developed using a TMB substrate kit (Pierce, Rockford, IL.) and the reaction was stopped by the addition of an equal volume of 1 M HCl. The results were read using a Spectramax colorimetric plate reader (Molecular Devices, Sunnyvale, CA).
Results

Microglia take up both soluble and fibrillar forms of Aβ

In order to elucidate the mechanism employed by microglia to take up soluble forms of Aβ, we derivatized sAβ with Cy3 or Alexa488 fluorescent chromophores. When these fluorescently labeled sAβ preparations were resolved on a 4-12% bis-tris gel, a majority (>90%) of sAβ was found to have an apparent molecular weight of approximately 4 kDa, and only low levels of more slowly migrating materials, corresponding to small Aβ oligomers, were observed. No fibrillization of the peptides was detected when sAβ was incubated at 2 µg/ml in culture medium for up to 18 hours and analyzed by SDS-PAGE (data not shown). Fluorescent labeling of Aβ allowed for the development of a FACS based assay that was used to monitor Aβ internalization by microglia. Previous studies have utilized immunofluorescence to monitor Aβ uptake; however, FACS has rarely been employed to characterize Aβ internalization and has allowed us to quantitatively measure the uptake of sAβ (Knauer et al., 1992, Ard et al., 1996, Paresce et al., 1997a, Chu et al., 1998, Chung et al., 1999, Koenigsknecht and Landreth, 2004, Simakova and Arispe, 2007).

We monitored Aβ uptake by microglia and found that both BV-2 microglia and primary microglial cells were able to take up soluble as well as fibrillar species of Cy3-Aβ (Figure 1). However, the subcellular distribution of the two species of Aβ was quite different. Soluble Aβ was localized in a diffuse pattern throughout the entire cell (Figure 1 A,C), in contrast to fAβ which was confined to large phagocytic vesicles within the cytoplasm (Figure 1 B,D). We evaluated the uptake of both Alexa488-labeled sAβ and fAβ using FACS analysis. Both BV-2 cells and primary microglia were able to take up
the labeled Aβ species at comparable levels. The intracellular levels of fibrillar Aβ were higher in both cell types in comparison to sAβ when similar levels of exogenous Aβ peptide were added to the culture medium (Figure 1E,F), although quantitative conclusions cannot be drawn owing to the different physical forms of the Aβ peptides. Importantly, the intracellular distribution of the peptides suggests that internalization of soluble and fibrillar Aβ occur through distinct mechanisms.

We verified that microglia in the living brain take up and compartmentalize sAβ. We monitored the internalization of sCy3-Aβ by microglial cells in vivo in a CXCR1/GFP+/+ mouse model (Figure 2). These mice possess microglial cells that express green fluorescence protein (eGFP). Fluorescent, Cy3 labeled Aβ was applied to the surface of the brain and its uptake into microglia was evaluated 3 hours later. We found that microglia internalize the sAβ peptides and traffic them into intracellular vesicles. The sAβ appears perinuclear in its intracellular distribution, similar to that in BV-2 cells and primary microglia. These data suggest that the mechanisms of sAβ uptake are similar both in vitro and in the living brain (Figure 2,B,C) and that microglia are proficient at clearing both soluble and fibrillar species of Aβ from the extracellular space.

**Soluble Aβ is not taken up through phagocytosis or receptor-mediated endocytosis**

To determine the mechanism of sAβ entry into microglia, we examined the kinetics of sAβ uptake. We found that Alexa488-labeled sAβ was taken into BV-2 cells in a time (Figure 3 A,B) and concentration (Figure 3 C) dependent manner. The uptake of the sAβ peptide appears to occur through a non-saturable mechanism, suggesting this process may be independent of cell surface receptors.
The cellular mechanisms responsible for internalization of sAβ peptides, until now, have not been well characterized. However, previous studies have shown that phagocytosis of fAβ requires the interaction of the Aβ fibrils with a cell surface receptor complex composed of the B-class scavenger receptor CD36, the α6β1 integrin and the integrin associated protein, CD47 (Bamberger et al., 2003, Koenigsknecht and Landreth, 2004). To determine whether this receptor complex plays a role in the internalization of sAβ, BV-2 cells were incubated with antagonists specific to the individual components of this receptor complex that serve to block fAβ-stimulated phagocytosis (Koenigsknecht and Landreth, 2004). Receptor antagonists were added to the culture medium 30 minutes prior to the addition of Alexa488-sAβ. CD47 is a transmembrane receptor that interacts with integrins and modulates integrin dependent signaling. Its activity is necessary for both interaction of fAβ with microglial cells as well as fAβ induced phagocytosis (Bamberger et al., 2003, Koenigsknecht and Landreth, 2004). 4N1K, an agonist peptide, is derived from the cell-binding domain of thrombosondin-1 (TSP-1), and interacts competitively with other CD47 ligands for CD47 binding (Chung, Gao & Frazier, 1997). Fucoidin inhibits both scavenger receptor class A and class B interactions (Husemann et al., 2002). Treatment of microglial cells with 4N1K or fucoidin did not inhibit uptake of sAβ by BV-2 cells. Treatment of microglial cells with 4N1K enhanced the uptake of sAβ into microglial cells. CD47 has been shown to interact with β-family integrins and modulate their response. Treatment of cells with 4N1K has been shown to promote cell spreading and migration in a number of cells via interaction with CD47. 4N1K acts to stimulate membrane ruffling and therefore increases uptake of sAβ by microglial cells (Chung et al., 1997, Brown and Frazier, 2001). A peptide antagonist of the β-class
scavenger receptor, CD36 (Frieda et al., 1995, Chung et al., 1997, Husemann et al., 2002) also failed to block the internalization of sAβ into the cells. We have previously shown that interactions between the microglial cell and fibrillar Aβ peptides is restricted to the same domains required for TSP-1 binding, which include amino acids 93-102 of CD36 (Coraci et al., 2002, Bamberger et al., 2003). The GST-CD36 peptide, is comprised of the extracellular binding domain of CD36 coupled to a GST tag, and blocks the interactions between CD36 receptor and other ligands (Frieda et al., 1995). The β1-integrin action was inhibited using INV195, a truncated form of invasin, a Yersinia protein that specifically binds this integrin subunit (Wiedemann et al., 2001) and blocks its interaction with fAβ (Koenigsknecht and Landreth, 2004). The full length form of invasin, Inv397, binds the β1-integrin and stimulates an intracellular signaling cascade inducing phagocytosis. This peptide was utilized to determine the role of this molecule in sAβ uptake (Isberg et al., 2000, Wiedemann et al., 2001). Neither inhibition, nor activation of the β1-integrin affected the internalization of sAβ into BV-2 microglial cells (Figure 4 A).

In addition to the fAβ receptor complex, the LDL receptor related protein-1 (LRP-1) has been implicated in the uptake and clearance of Aβ (Wiedemann et al., 2001, Deane et al., 2004, Harris-White and Frautschy, 2005, Burns et al., 2006a, Sagare et al., 2007). LRP plays a role in the internalization as well as the degradation of lipoproteins and has been shown to mediate fAβ endocytosis (Harris-White et al., 2004). To test the role of LRP in uptake of sAβ, BV-2 microglial cells were incubated with the LRP antagonist, receptor associated protein (RAP), for 30 minutes prior to the addition of Alexa488-sAβ. RAP chaperones the folding of LRP and has been shown to compete strongly with all
other LRP ligands, functionally antagonizing the actions of this receptor (Iadonato et al., 1993). When assessed by flow cytometry, blocking LRP with RAP had no effect on sAβ internalization (Figure 4 B) by microglia.

An alternate means through which cells can internalize solutes from the extracellular milieu is through clathrin-mediated endocytosis. The uptake of transferrin has been shown to occur through this mechanism (Bleil and Bretscher, 1982). To determine if sAβ is internalized through a clathrin-coated vesicle mechanism, co-localization studies were carried out using Alexa488-labeled transferrin and Cy3-sAβ. We found no colocalization between transferrin and sAβ within early endosomes (Figure 4 C), indicating that initial internalization of the proteins occurs via distinct subpopulations of vesicles. Together, these data suggest that sAβ is not taken up into microglial cells through a receptor-mediated mechanism. To verify this conclusion, a competition assay was performed. BV-2 cells were treated for 3 hours with 2 µg/ml of Alexa488-sAβ in the presence of increasing concentrations of unlabeled sAβ peptides. The samples were then fixed and analyzed by flow cytometry. If sAβ is internalized via a receptor-dependent mechanism, then the addition of unlabeled sAβ should compete with the uptake of the fluorescently labeled sAβ species. We demonstrate that increasing levels of unlabeled sAβ had no effect on the uptake of fluorescent sAβ by microglial cells as observed by FACS analysis (Figure 5). These data suggest that the mechanism of sAβ uptake occurs through a non-saturable, fluid phase mechanism.

**Microglia take up sAβ through fluid phase macropinocytosis**

Fluid-phase pinocytosis includes two distinct mechanisms, macropinocytosis and micropinocytosis. Macropinocytosis results in the formation of vesicles that are between
0.2 µm and 5.0 µm in diameter and are created by the enclosure of membrane ruffles. Micropinocytosis occurs via the formation of vesicles within cholesterol-rich lipid domains. These vesicles can also be caveolin-coated (Nichols, 2003). To determine the route of sAβ entry, BV-2 cells were treated with nocodazole and cytochalasin D, which inhibit tubulin depolymerization and actin polymerization respectively. Both cytoskeletal structures are necessary for the formation of membrane ruffles and the subsequent formation of macropinosomes (Conner and Schmid, 2003, Wehrle-Haller and Imhof, 2003). Treatment of BV-2 cells with both of these agents significantly reduced uptake of sAβ by BV-2 cells, as measured by flow cytometry (Figure 6 A,B). However, treatment of cells with filipin, which depletes membrane cholesterol and inhibits micropinocytosis from lipid domains (Fujita et al., 1981), did not affect sAβ internalization (Figure 6 C). These data indicate that sAβ enters microglial cells via fluid phase macropinocytosis.

Once internalized, sAβ is rapidly trafficked into the lysosomes via the endolytic pathway. Colocalization between Cy3-sAβ and Lysotracker was seen within 15 minutes of Aβ peptide treatment in BV-2 cells using real time imaging techniques (Figure 7 D, Supplemental video 1 and 2). Previous work has shown that fAβ is trafficked to the lysosomes after phagocytosis (Paresce et al., 1997a, Majumdar et al., 2007). Trafficking of sAβ appears to occur by transfer to the late endosomes and lysosomes by direct fusion of the macropinocytic vesicle to these late endolytic vesicles. Soluble Aβ colocalizes directly with Lamp1/Lamp2 (Figure 7 A, B), markers of late endolytic pathway, but not with Rab5b, a marker of early endosomes (Stein et al., 2003) (Figure 7 C). Together, these data demonstrate that sAβ enters microglial cells via fluid phase macropinocytosis, a mechanism that is distinct from classic receptor-mediated endocytic pathways.
Soluble and fibrillar species of Aβ localize to different intracellular compartments

Consistent with a pinocytic uptake mechanism, sAβ was colocalized with 20nm microspheres which are commonly used to monitor pinocytic uptake (Falcone et al., 2006) (Figure 8 A). In addition, the 1 µm beads taken up through phagocytic mechanisms were rarely found to colocalize with the 20 nm microspheres, consistent with their different mechanisms of uptake (Figure 8 B,D). However, the 20 nm microspheres, given their small size, can also enter phagocytic vesicles and, in some cases, can be seen partially colocalized with the 1 µm beads (Figure 8B, C). Importantly, Alexa488-sAβ and Cy3-fAβ are largely localized to different cellular compartments after being taken up by BV-2 cells. We found a fraction of sAβ colocalized to vesicles carrying fAβ suggesting some sAβ enters phagocytic vesicles and can also be taken up when this mechanism is active owing to bulk uptake of extracellular fluid upon phagosome formation (Figure 8E,F). The largely distinct subcellular distribution of both fibrillar and soluble Aβ peptides is evidence of their different modes of entry into the cell, through a phagocytic mechanism and fluid phase macropinocytosis, respectively.

Uptake of sAβ by other cell types

The macropinocytic uptake of sAβ is reflective of the capacity of the various cell types to form membrane ruffles. The closure of these membrane ruffles results in the formation of endocytic vesicles allowing the cells to non-specifically take up the contents of the extracellular milieu. In addition to microglia, both neurons and astrocytes have the capacity to internalize Alexa488-sAβ (Figure 9B, C, F, G). Quantitative analysis has revealed that microglial cells take up Alexa488-sAβ much more efficiently than other cell types under the same conditions (Figure 9D). Astrocytes are heterogeneous with
respect to their ability to take up Aβ, one population of cells can internalize sAβ just as efficiently as microglial cells while another population does not take up any Aβ at all (Figure 9 F). The basis of this effect is presently unclear. Mouse embryonic fibroblasts (MEFs), a cell type not found in the CNS can also efficiently internalize the sAβ peptide (Figure 9A) and has an uptake profile similar to astrocytes. The subcellular distribution of sAβ in all three cell types is quite similar to that observed in microglia, with Aβ distributed within vesicles throughout the cytoplasm (Figure 9A, B, C).

**Fluorescent labeling of sAβ does not alter its susceptibility to proteolysis**

The fluorescently-labeled Aβ species have been useful in examining Aβ uptake and trafficking, but we found they they have limited utility in examining the subsequent clearance of the peptide by microglia. To determine if fluorescently-labeled Aβ was susceptible to proteolytic degradation, the internalization as well as degradation of labeled and unlabeled peptides were measured using flow cytometry and ELISA respectively. BV-2 cells were incubated with 2µg/ml Alexa488-labeled or unlabeled Aβ peptides for 3 hours then the media was removed and replaced with serum-free DMEM. Cells were incubated for another 3 hours and then monitored for intracellular levels of sAβ.

We found that unlabeled and Alexa488 conjugated Aβ preparations were taken up at equivalent levels by BV-2 (Figure 10A) cells. Importantly, all species of sAβ, either labeled or unlabeled, were degraded efficiently, as measured by ELISA. We found that approximately 50% of the internalized sAβ was degraded following 3 hour incubation in either BV-2 cells (Figure 10B) or primary microglia (Figure 10C). In control studies, Aβ levels in the media were also monitored to control for resecretion of the peptide back into
the media; however, no Aβ was detected in the media (Figure 10D). Interestingly, when FACS analysis of the Alexa488-sAβ conjugated peptide was carried out, we did not observe a parallel decrease in intracellular fluorescent intensity (Figure 10E). Similarly, the fluorescent intensity of fixed cells that internalized Cy3 conjugated sAβ did not diminish following washout despite the fact that over 50% of the labeled protein was degraded when Aβ levels were measured by ELISA (Figure 10F). These data may resolve the controversial findings reported by Paresce et al. (1997), who observed that microglia remain fluorescent following uptake of fibrillar Cy3-Aβ for up to 6 days in culture. This was interpreted as an inability of microglia to degrade fAβ, but could be explained by retention of the fluorophore within the cell. The unconjugated Cy3 or Alexa488 fluorophores are not taken up by microglial cells (data not shown); therefore, all fluorescent signal observed intracellularly is reflective of internalized sAβ peptides. The fluorescent derivitization of the peptide, therefore, serves as an excellent tool to examine Aβ uptake, but cannot be employed to evaluate Aβ degradation.
Discussion

The response of microglial cells to amyloid deposition has been extensively studied in Alzheimer’s disease. Fibrillar forms of Aβ arise from the polymerization of the soluble, monomeric or oligomeric forms of the Aβ peptide. Despite the abundance of activated microglia, they are inefficient in clearing fAβ deposits (Rogers and Lue, 2001, Bolmont et al., 2008). Thus, the regulation of sAβ levels is a critical determinant in the development of AD pathology. This manuscript focuses on how microglia, one of the many cell types found in the CNS, participates in the maintenance of Aβ homeostasis. We show that microglia clear sAβ species through the process of macropinocytosis both in vitro and in the living brain.

The levels of Aβ peptides within the brain are tightly regulated by mechanisms controlling their generation and clearance (Tanzi & Bertram, 2005). In the brain, approximately 8% of total Aβ is synthesized per hour and cleared at a roughly equal rate, thus, preventing its accumulation and deposition in the normal brain (Bateman et al., 2006). However, modest perturbations in Aβ clearance will result in accumulation of sAβ peptides within the brain and their subsequent deposition into plaques. It is possible that the inflammatory environment, characteristic of the AD brain, may affect the ability of microglia to macropinocytose sAβ from the milieu, acting to promote disease pathogenesis.

Macropinocytosis is a common mechanism employed by many cell types for the bulk phase uptake of fluids and nutrients from the environment. This process occurs through the closure of membrane ruffles creating vesicles capable of entering the endolytic
pathway (Swanson and Watts, 1995, Falcone et al., 2006). Macropinocytosis has been extensively studied in dendritic cells which use this pathway to take up proteins for antigen presentation (Sallusto et al., 1995, Norbury, 2006) and in Dictyostelium, which employs macropinocytosis to internalize fluids and nutrients in bulk phase (Cardelli, 2001, Amyere et al., 2002). Macropinocytosis has also been implicated in a number of disease mechanisms. For example, macrophages utilize this mechanism to take up lipids leading to the formation of foam cells (Kruth et al., 2005). Most recently, it has been reported that macropinocytosis mediates HIV-1 infection in trophoblastic cells (Marechal et al., 2001, Vidricaire and Tremblay, 2007). Depending on the cell type, macropinocytosis can be constitutive or induced. Resting ramified microglia in rat brain slices, as well as in tissue culture exhibit high levels of constitutive pinocytic activity (Ranson and Thomas, 1991).

Our results illustrate that microglial cells can internalize sAβ by constitutive, non-saturable, fluid phase macropinocytosis both in vitro and in vivo (Figure 2). We have ruled out the involvement of receptor-mediated endocytic processes as well as phagocytosis in sAβ uptake by microglia. Inhibition of fAβ receptor components as well as LRP had no effect on sAβ uptake. Furthermore, competition assays using increasing concentrations of unlabeled sAβ peptides did not affect internalization of fluorescently labeled peptide, demonstrating this process is non-specific, non-saturable and not receptor dependent (Figure 5). These findings are consistent with our failure to find colocalization of sAβ with transferrin, which is taken up through receptor-mediated, clathrin coated, endocytosis. These studies indicate that fAβ receptor complex, LRP or other receptor-mediated forms of endocytosis do not play a role in sAβ internalization.
Macropinocytosis is dependent on both actin and microtubule dynamics which are necessary for the formation of membrane ruffles and the subsequent development of macropinocytic vesicles (Chhabra and Higgs, 2007, Gao et al., 2007). Consistent with the idea that sAβ is taken up by microglia through this mechanism, inhibition of actin polymerization (Cytochalasin D) or preventing the depolymerization of microtubules (nocodazole) significantly reduced accumulation of sAβ intracellularly (Figure 6A, B). This pathway is distinct from micropinocytosis which forms small vesicles less than 0.1 μm in diameter and occurs in cholesterol-rich lipid raft domains (Cardelli, 2001). Inhibiting micropinocytosis by cholesterol depletion using filipin had no effect on sAβ uptake (Figure 6C).

Internalized sAβ is rapidly delivered to the lysosomes via the late endolytic pathway. Direct uptake of particles into late endolytic vesicles has been shown to be a characteristic of macropinocytosis in Dictyostelium (Cardelli, 2001). These vesicles are Lamp1/2 positive and negative for Rab5b (Figure 7A, B, C). This process is rapid and accumulation and colocalization of the sAβ can be seen by real time imaging in lysotracker-positive vesicles within minutes of exposure (Figure 7D, supplemental videos). Interestingly, this pathway is characteristic of soluble, but not fibrillar Aβ uptake. Colocalization studies showed that sAβ and fAβ are initially trafficked to different compartments within the cells, suggesting that distinct mechanisms of entry are utilized by different species of Aβ (Figure 8). This observation is consistent with previous work which demonstrated that microglia segregate different sized fluorescent dextran beads into distinct endo-lysosomal vesicles based on their size (Berthiaume et al., 1995). Once internalized, Aβ microaggregates, have been shown to colocalize to cellular
fractions containing the lysosomal markers β-hexosaminidase and acid phosphatase (Knauer et al., 1992). Furthermore, these microaggregates exhibit perinuclear distribution and colocalize to vesicles containing α2M, indicating their trafficking into lysosomal vesicles (Knauer et al., 1992, Paresce et al., 1997a, Chung et al., 1999). In a recent study, using two-photon imaging, microglial cells were shown to play a role in the plaque dynamics in an AD transgenic mouse model. Microglia were observed to internalize plaque fragments and deliver them to the lamp2-positive vesicles (Bolmont et al., 2008). These data suggest that even though the initial uptake of sAβ and fAβ occurs through distinct mechanisms both species are ultimately trafficked to the lysosomes. However, since macropinocytic vesicles range in size from 0.5 to 5 µm in diameter, it is possible that larger oligomeric species of Aβ are internalized via macropinocytosis as well.

Importantly, this study demonstrates that labeling of Aβ peptides with a fluorophore is a valuable tool to measure cumulative Aβ uptake by microglial cells. It must be emphasized; however, that the conjugation of a fluorescent chromophore to this particular peptide does not alter its intracellular degradation. We observed that the labeled sAβ protein was proteolytically degraded with the same efficiency as the unlabeled peptide (Figure 10B, C). This latter finding is inconsistent with a previous report that over 80% of internalized sAβ is resecreted back into the culture medium by microglia within 10 hours of initial uptake (Chung et al., 1999). Under similar conditions, we observed no reserecreation of the peptide into the media as measured by ELISA (Figure 10D).

Paresce and colleagues have shown that Cy3-labeled microaggregates of Aβ were internalized by microglial cells and reported that they were resistant to degradation for over six days in culture, leading to the conclusion that microglia cannot degrade fAβ
(Paresce et al., 1997a). In contrast, our data demonstrates that the labeled soluble peptide is intracellularly degraded; however, the fluorescent chromophore is retained by the cells and can be detected using both flow cytometry as well as in fixed cells. (Figure 10C, D). These findings provide a possible explanation for the persistent fluorescent signal attributed to the Cy3 microaggregates observed by Paresce et al. The use of fluorescently labeled Aβ species, are a useful tool to evaluate Aβ uptake and trafficking; however, caution must be exercised when using these modified proteins to evaluate their degradation.

Recently, a debate has arisen over the role and ability of bone marrow derived microglia to cross the blood brain barrier (BBB) and clear Aβ in the brain. Simard and colleagues have reported that these peripherally derived cells transit the BBB and are capable of clearing Aβ deposits from the brain, whereas resident CNS microglia have an impaired ability to phagocytose Aβ (Simard et al., 2006). However, two recent publications have shown that under physiological conditions, peripherally derived monocytes and macrophages are unable to cross the BBB. The ability of microglia to enter the CNS was shown to be an artifact of the irradiation and reconstitution of bone marrow of the host animal (Ajami et al., 2007, Mildner et al., 2007a).

In addition to microglia, both astrocytes and neurons have been postulated to play a role in Aβ clearance. Adult, but not neonatal, mouse astrocytes have been shown take up and degrade fibrillar Aβ deposits from brain slices of transgenic animals expressing the human amyloid precursor protein. Uptake of Aβ deposits into astrocytes was demonstrated to be dependent on scavenger receptors as well as ApoE (Wyss-Coray et al., 2003, Koistinaho et al., 2004). It has been suggested that the uptake of small
oligomeric species of Aβ by neurons occurs through a caveolae-independent, lipid raft-dependent mechanism on distal axons and is transported to the cell body in a retrograde fashion (Saavedra et al., 2007). Another study showed that the internalization of sAβ is facilitated by the α7 nicotinic acetylcholine receptor in both brain slices and neuronal cultures (Nagele et al., 2002). However, the precise mechanism of internalization remains to be elucidated. Importantly, while neurons and astrocytes have been shown to have the ability to take up Aβ, it is unclear whether they have the capacity to degrade this peptide.

In the present study we have demonstrated that internalization of sAβ is not limited to microglia, as both astrocytes and neurons are capable of taking up sAβ as assessed by flow cytometry (Figure 9). Since macropinocytosis is a ubiquitous process and each cell has the capacity to pinocytose, this process is not unique to microglial cells and may play a more widespread role in the clearance and homeostasis of sAβ in the brain by multiple cell types. However, our studies demonstrate that microglial cells are the most efficient at this process in vitro, internalizing almost four times more sAβ than astrocytes (Figure 9D). Additionally, we show that microglia are also efficient at degrading sAβ (Figure 10). These studies characterize the mechanism microglia employ in the clearance of sAβ peptides, suggesting they may play a more abundant role in the maintenance of Aβ homeostasis in the brain.
Figure 1: Microglial cells can take up both soluble and fibrillar species of Aβ. BV-2 microglial cells (A,B,E) and primary microglial cultures (C,D,F) were incubated with 2 µg/ml Cy3-labeled soluble (A,C) or fibrillar (B,D) forms of Aβ42 for three hours. Cells were then fixed, permeabilized and stained with DAPI. Uptake was quantified using flow cytometry on the EPICS-XL MCL in BV-2 cells (E) as well as primary microglia (F) and compared to control non treated cells (black trace).
Figure 2: Microglia cells internalize sCy3-Aβ in vivo. A craniotomy was performed on a CX3CR1/GFP+/+ mice and the brain was exposed to 1-2 µg of sCy3-Aβ. After 3 hours of exposure to the peptide the mouse was perfused and the brains isolated and sectioned. Confocal images were obtained using the 20x (Microglia that have taken internalized sCy3-Aβ are boxed in white) (A) and 100x (B,C) objectives. Confocal images of microglial cells that have internalized sCy3-Aβ are shown in the x and y planes (C).
Figure 3: Time course and dose-dependence of sAβ uptake. BV-2 cells were incubated for the indicated times with 2 µg/ml soluble-Alexa488-Aβ, fixed and analyzed by flow cytometry on the EPICS-XL MCL (A, B). BV-2 cells were incubated for 3 hours with increasing concentrations of soluble Aβ, cells were lysed and intracellular Aβ levels were evaluated by ELISA (C).
Figure 4: sAβ uptake is not mediated by the fAβ receptor complex or receptor-mediated endocytosis. BV-2 cells were incubated with the indicated inhibitors for 30 minutes prior to three hour incubation with 2 µg/ml soluble Alexa488-Aβ. The cells were then fixed and uptake was analyzed using flow cytometry on the EPICS-XL MCL (A). Cells were also treated with inhibitors of LRP and ABCA1, both of which have been linked to Aβ clearance (B). BV-2 cells were treated with Cy3-Aβ and transferrin-Alexa488, which has been shown to be taken up through clathrin mediated endocytosis, for one hour and fixed. Cells were imaged using a Zeiss 510 confocal microscope (C).
Figure 5: Microglia take up sAβ through a non-saturable mechanism. BV-2 cells were incubated for 3 hours with 2 µg/ml of soluble Alexa488-Aβ in the presence of the indicated amounts of unlabeled soluble Aβ peptide. Cells were then fixed and analyzed using flow cytometry on the EPICS-XL MCL.
Figure 6: Microglial cells internalized sAβ through macropinocytosis. BV-2 cells were incubated with nocodazole, an agent that disrupts microtubule dynamics (A) and cytochalasin D, an inhibitor of actin polymerization (B) or filipin, a cholesterol-depleting agent (C) for 30 min prior to the addition of soluble Alexa88-sAβ for 3 hours. Cells were then fixed and analyzed using flow cytometry on the EPICS-XL MCL.
Figure 7: Upon internalization sAβ is trafficked into late endosomes and lysosomes.

BV-2 microglia were incubated with 2 µg/ml sCy3-Aβ for 3 hours, after which they were fixed. Lysosomes were visualized using Lysotracker or LAMP1/2 (markers of late endosomes and lysosomes) (A,B) and early endosomes were labeled using Rab5B. (C) Cells were then imaged on a Zeiss 510 confocal microscope. Real time imaging was carried out using Lysotracker (lysosomal marker) and soluble Cy3-Aβ (D).
Figure 8: Soluble and Fibrillar Aβ are internalized through distinct mechanisms.
BV-2 microglia were incubated with soluble Cy3-Aβ and 20nm fluorescent microspheres (green) for 1 hr. The cells were then fixed and visualized. Uptake of 20 nm microspheres colocalized with sCy3-Aβ (A) the colocalization is also visible in the x and y planes (D). Uptake of 1 µm beads (red) occurs through phagocytosis. When both microspheres and 1 µm beads were co-incubated they showed distinct sub-cellular distributions (B) indicating different mechanisms of uptake. Confocal images of BV-2 cells that have internalized 1 µm beads as well as 20 nm microspheres from panel B are shown in the x and y planes (C). Fibrillar Cy3-Aβ, which is taken up through phagocytosis and soluble Alexa488-Aβ, which is taken up through pinocytosis were co-incubated for one hour prior to fixation (D) there distribution in the x and y planes (F).
Figure 9: **Internalization sAβ is not unique to microglia.** Astrocytes, neurons, primary microglia or MEFs were plated at a density of 800,000 cells/well of a six well plate and incubated with 2 µg/ml of soluble Cy3 labeled Aβ or Alexa488 labeled Aβ peptide for 6 hours. The internalization of sAβ was visualized using the Leica DMIRB research microscope (A,B,C) or by flow cytometry on the EPICS-XL MCL(D, E, F, G, H). The cells were then fixed and permeablized, stained with DAPI and phalloidin and mounted on coverslips. The mean fluorescent intensity of internalized Aβ, is shown (D) and specific values are as follows; 28.29 (Microglia), 7.56 (Astrocytes), 4.71 (Neurons) and 13.67 (MEFs).
Figure 10: Microglial cells proteolytically degrade fluorescently labeled sAβ upon internalization leaving behind the fluorescent chromophore. BV-2 microglial cells were incubated with 2 µg/ml of soluble Alexa488 labeled or unlabeled Aβ peptide. The levels of internalized sAβ was determined using ELISA (A). The degradation of the labeled or unlabeled Aβ peptides was monitored using ELISA in both BV-2 microglial cells (B) and primary microglia (C). The cells were allowed to internalize sAβ (labeled or unlabeled peptides) for 3 hours (black bars) parallel cultures were washed and incubated for an additional 3 hrs in medium lacking sAβ (striped bars). Intracellular Aβ levels were evaluated using ELISA (B,C), flow cytometry on the EPICS-XL MCL (E) and immunofluorescence of Cy3 labeled Aβ (F). The black traces in (E) represent control non treated cells. In addition, levels of Aβ in the media were monitored to control for resecretion of the peptide (D).
Chapter 3

PPARɣ activation rapidly ameliorates amyloid pathology and restores cognition in a mouse model of Alzheimer’s disease

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Abstract

Alzheimer’s disease is associated with a disruption of Aβ homeostasis, resulting in the accumulation of Aβ peptides within the brain and their deposition into diffuse and dense core amyloid plaques. Peroxisome proliferator-activated receptor-γ (PPARγ) is a ligand-activated nuclear receptor that acts primarily to regulate lipid and glucose homeostasis, but also, exhibits potent anti-inflammatory actions and is found in elevated levels in the AD brain. We show that activation of PPARγ with the synthetic agonist, pioglitazone (Actos™), enhances the proteolytic degradation of Aβ in both microglia and astrocytes in vitro. We demonstrate that this is accomplished through the participation of PPARγ in a coupled metabolic pathway with Liver X Receptors that results in the elevation of Apolipoprotein E levels. The proteolytic degradation of Aβ occurs though an apoE dependent mechanism that is reliant on the induction of LXRs by PPARγ. A nine day treatment of APPswe/PS1Δe9 mice with pioglitazone, resulted in dramatic reductions in brain levels of soluble and insoluble Aβ. Importantly, pioglitazone treatment suppressed glial activation, reversed AD related cognitive deficits and altered the phenotypic polarization of microglial cells. Additionally, 12 month old APPswe/PS1Δe9 animals exhibited abundance Aβ-laden microglia and astrocytes in cortex after pioglitazone treatment. Together, our data provides a mechanistic explanation for how PPARγ activation facilitates amyloid clearance and validates the therapeutic utility of PPARγ agonists for the treatment of Alzheimer’s disease.
Introduction

Late onset Alzheimer’s disease (LOAD) is characterized by the progressive accumulation of amyloid beta (Aβ) within the cortex and hippocampus. Aβ levels in the CNS are strictly regulated by mechanisms controlling the generation and clearance of this peptide (Tanzi and Bertram, 2005, Kim et al., 2009). In a healthy brain, approximately 8% of total Aβ is generated per hour and clearance of the peptide occurs at a similar rate, allowing for the proper maintenance of Aβ homeostasis (Bateman et al., 2006).

Importantly, Mawuenyega et al have reported that in LOAD-patients, Aβ clearance is reduced by approximately 25%, owing to age related impairments in Aβ clearance mechanisms (Mawuenyega et al., 2010) AD-related accumulation and deposition of these peptides in the brain has been associated with a robust microglial-mediated inflammatory response, perturbation of in synaptic function, extensive neuronal loss and behavioral deficits (Lue et al., 1999, Akiyama et al., 2000, Lacor et al., 2007). The “classical” proinflammatory, or “M1” activation, of microglia is postulated to contribute to disease pathogenesis and neuronal loss.

The apolipoprotein E (ApoE) gene is the principal risk factor for sporadic, late onset cases of Alzheimer’s disease (Roses, 1996). In the brain, apoE is primarily synthesized by astrocytes, however, microglia express ApoE at lower levels (Pitas et al., 1987, Grehan et al., 2001). ApoE plays a critical role in modulating levels of Aβ peptides in the brain by controlling Aβ fibrillogenesis, transport and clearance (Holtzman, 2001, Zlokovic et al., 2005, Kim et al., 2009). ApoE acts to scaffold the formation of high density lipoproteins (HDL), which act to traffic cholesterol and
phospholipids throughout the brain (Kim et al., 2009). The transfer of phospholipids and cholesterol to apoE-containing HDL particles occurs primarily through the actions of the cholesterol transporter, ATP-binding cassette transporter A1 (ABCA1), (Hirsch-Reinshagen et al., 2004, Wahrle et al., 2005). Jiang et al. demonstrated that ApoE-containing HDL particles act to facilitate the proteolytic degradation of Aβ, both in the interstitial fluid and by glia. The lipidation status of ApoE is a critical determinant governing the efficiency of this process. The larger the HDL-particle size the more effectively soluble species of Aβ are cleared from the brain (Jiang et al., 2008). Several studies have also demonstrated the necessity of ABCA1 activity for the effective clearance of Aβ in mouse models of AD (Hirsch-Reinshagen et al., 2005, Koldamova et al., 2005a, Wahrle et al., 2005, Wahrle et al., 2008, Donkin et al., 2010, Fitz et al., 2010).

Nuclear receptors comprise a family of ligand-activated transcription factors and include the peroxisome proliferator-activated receptor-γ (PPARγ) and Liver X receptor (LXR). PPARγ acts as a whole body lipid sensor and plays a dominant role in lipid homeostasis, insulin sensitivity and energy metabolism (Forman et al., 1997). LXRs act as cholesterol sensors and their activation has been shown to induce the expression of the reverse cholesterol transport genes, apoE and ABCA1. The activation of both PPARγ and LXRs results in amelioration of AD related pathology and behavioral impairments in a mouse models of AD (Koldamova et al., 2005b, Riddell et al., 2007, Zelcer et al., 2007, Jiang et al., 2008, Donkin et al., 2010, Fitz et al., 2010) In the periphery, LXRs and PPARγ participate in a coupled metabolic pathway coordinately regulating lipid metabolism (Chawla et al., 2001), whereby activation of PPARγ induces the expression of LXRα and its target genesAbca1 and ApoE. Conversely, LXR activation induces
PPAR\(\gamma\) expression, metabolically linking these two pathways (Chawla et al., 2001, Seo et al., 2004, Yue and Mazzone, 2009). However, these relationships have not previously been explored in the brain.

Recently, PPAR activation has been appreciated in playing a role in promoting the acquisition of an M2 or “alternative” phenotype in peripheral macrophages, that provide an anti-inflammatory balance, by suppressing the expression of inflammatory cytokines, and promoting the expression of proteins thought to play a role in wound healing and inducing phagocytosis (Bouhlel et al., 2007, Odegaard et al., 2007, Gallardo-Soler et al., 2008, Odegaard et al., 2008, Chawla, 2010). This is in part achieved by their ability to suppress NF\(\kappa\)B mediated inflammatory gene induction (Kersten et al., 2000, Combs et al., 2001a, Heneka and Landreth, 2007). Alternative activation states of macrophages have been studied in detail in the periphery however, while they are known to be expressed in the brains of AD patients and mouse models the mechanisms that modulate these phenotypic changes in the brain remain unknown (Colton et al., 2006, Jimenez et al., 2008, Lee et al., 2010). It is unknown if PPAR agonists can affect microglial phenotype in the brain.

We demonstrate that activation of PPAR\(\gamma\), using the synthetic agonist pioglitazone (Actos\textsuperscript{TM}), induces the expression of LXR\(\alpha\) and its target genes, apoE and ABCA1, elevates brain HDL levels and promotes A\(\beta\) clearance mechanisms in both microglia and astrocytes. Genetic inactivation of PPAR\(\gamma\) within the brain reduces apoE and Abca1 expression. This study provides the first evidence for a metabolic link between the PPAR and LXR pathways in the brain. Moreover, the treatment of APP/PS1 mice with pioglitazone (80mg/kg/day) for nine days results in the rapid reduction of A\(\beta\)
peptides and plaque levels in the brain and is associated with improved contextual memory in 12 month old APP/PS1 mice. We demonstrate that PPARγ activation changes the inflammatory milieu in the brain, converting the “classical” inflammatory (M1) milieu associated with AD, to an alternative “M2” phenotype, associated with the robust phagocytosis of amyloid plaques by both microglia and astrocytes. These data provide a mechanistic link between PPARγ activation and amyloid clearance in the AD brain and support the therapeutic use of its agonists in AD.
Materials and Methods

Reagents-Aβ1-42 peptide was purchased from American Peptide Company, dissolved in DMSO for a final concentration of 1μg/ml.

Cell Culture-Primary microglia and astrocytes were cultured from post-natal day 0-3 C57BL/6J mice and previously described (McDonald et al., 1997). Briefly, pups were decapitated and the meninges and blood vessels were removed from the cortex. The cortices were then minced and trypsinized with 0.05% trypsin-EDTA (Gibco) for 20 min at 37°C. DMEM/F12 (Gibco) containing 10% heat-inactivated fetal bovine serum (HI-FBS, Atlanta Biologicals) and 1% penicillin-streptomycin (Gibco) were then added to stop trypsin digestion. Cells were then triturated and plated in 150mm dishes (Gibco) at a density of half a cortex/plate. Media was changed the following day to fresh DMEM/F12 with 10% HI-FBS and 1% pen/strep for 14-21 days and were incubated at 37°C, 5% CO2. Microglia were isolated as described by Saura et al. (Saura et al., 2003). Briefly, the microglia were removed by shaking the tissue culture plates to remove loosely adherent microglia for 30 min. Astrocytes were removed next by 0.25% trypsin/HBSS diluted 1:3 in serum free F12/DMEM at 37°C for 30 min. The astrocytes were then collected by centrifugation at 2000 rpm for 5 min. The cells were then resuspended in F12/DMEM containing 10% HI-FBS and plated in 24 or 6 well plates (Gibco). Astrocytes were allowed to grow for 3-5 days prior to experiments. Firmly attached microglia were harvested using 0.25% trypsin/PBS. Loosely and firmly adherent microglia were then combined and cells were counted and plated for experiments at the appropriate densities
in DMEM/F12 media containing HI-FBS. Media was changed to serum free DMEM/F12 one night prior to beginning experiment.

*Αβ ELISAs*—Αβ levels were quantified using an Αβ₄₂ specific antibody, 6E10, as the capture antibody and monoclonal 4G8 HRP-conjugated antibodies (Covance). Synthetic Αβ₄₂ was used to generate a standard curve for each experiment. The plates were developed using the TMB substrate kit (Pierce), and the reaction was stopped by the addition of 1 M HCL. The results were read using a Spectramax colorimetric plate reader (Molecular Devices). Primary mouse microglia or astrocytes were incubated with DMSO or relevant concentrations of pioglitazone (dissolved in DMSO), T0070907 (10nM), 22S hydroxycholesterol (10μM) for 24 hours at 37°C. Cells were than treated with 2μg/ml soluble Αβ₄₂ in serum free media for 24 hours. Purified human plasma ApoE (rPeptide) or ApoAI was applied at the same time as soluble Αβ₄₂. Cells were washed with PBS and lysed in 1% SDS. Αβ₄₂ levels were measured using ELISA and normalized to total protein.

*Western blot analysis*—Protein concentration of cell lysates or brain extracts were measured using the BCA method (Pierce). Bis-Tris 4-12% gels (Invitrogen) were used. The following primary antibodies were used: anti-human Aβ, 6E10 (Covance); anti-ApoE; anti-β-actin (Santa Cruz); anti-ABCA1 (Novus Biologicals).

*Animals*—APPswe/PS1Δe9 (APP/PS1) transgenic mice [B6C3-Tg(APPswe,PSEN1dE9)85Bdo/J] were obtained from Jackson Laboratories (Jankowsky et al., 2004). APP/PS1 mice or wildtype littermates were gavaged daily for 9 days with 80mg/kg/day of pioglitazone or vehicle (water). Floxed PPARγ (PPARγfl/fl) mice
[B6.129-Pparg^tm2Rev] were obtained from Jackson Laboratory and were crossed with GFAP-cre mouse [B6.Cg-Tg(Gfap-cre)73.12Mvs/J] also obtained from Jackson Laboratories, to generate mice that lacked PPARγ expression in astrocytes and cortical neurons. The animals were then sacrificed and one hemisphere was fixed and processed for immunohistochemistry. The hippocampus and cortex were removed from the other hemisphere and snap-frozen and stored at -80 until they were subject to RNA and protein extraction. All experiments involving animals followed approved protocols by the Case Western Reserve University School of Medicine.

_Tissue collection and immunohistochemistry_ – Postfixed hemispheres were sections into 10μm sections using a cryostat. Sections were mounted, air-dried, and then stored at -20°C until use. thioflavin S (Sigma) staining was performed by rehydrating sections and staining with 1% thioflavin S. Slides were coverslipped with Prolong gold (Invitrogen). The numbers of thioS+ plaques were counted by a blinded observer. For 6E10, sections were pretreated with 70% formic acid for 3 min. Sections were then blocked with 5% normal goat serum and incubated overnight in primary antibody (6E10, 1:1000 (Signet Laboratories), Iba1, 1:300 (Wako), CD45, 1:500 (Serotec), GFAP, 1:1000 (Dako). Slides were incubated with the appropriate Alexa-conjugated secondary antibodies, followed by DAPI labeling of nuclei. Two sections per slide and 3 slides per animals, spaced evenly from 200μm anterior to the appearance of the CA3 to the end of the hippocampus, were analyzed. Images were analyzed for the percent area occupied by 6E10 positive amyloid plaques using Image Pro-Plus software (Media Cybernetics).
Brain homogenates and Aβ ELISA – Cortices were removed from hemibrains and were homogenized in 800 ml of tissue homogenizing buffer (THB; 250 mM sucrose, 20mM Tris, 1mM EDTA, 1mM EGTA in DEPC water) containing protease inhibitor cocktail (1:100, Sigma) using a glass-on-glass homogenizer at 4°C. The homogenate was centrifuged at 5,000 x g for 10 min at 4°C and supernatants were collected and stored at -80°C for western blot analysis. For “soluble” Aβ extraction 400μl of homogenates were mixed with 400 μl of 0.4% diethylamine, 100 mM NaCl, and the samples were homogenized using a glass-on-glass homogenizer. Samples were centrifuged at 135,000 x g for 1 hr at 4°C. Supernatants were collected, 0.5M Tris-HCl, pH 6.8 was added and the samples were stored at -80°C for analysis. The resulting pellet was sonicated in 70% cold formic acid and centrifuged at 109,000 x g for 1 hr at 4°C. The supernatant was collected, the formic acid was neutralized, and the samples were stored at -80°C for the analysis of “insoluble” Aβ. Aβ1-40 and Aβ1-42 ELISAS were performed using 6E10 as the capture antibody and HRP-conjugated antibodies specific to Aβ1-40 and Aβ1-42 (Covance) for detection and processed as described above. Samples were normalized to protein concentration using the BCA method.

RNA extraction, reverse transcription and quantitative PCR – Total RNA was isolated from cortices using RNA-Bee (Tel-Test, Inc.). Equal amounts of the homogenate and RNA-Bee were combined, chloroform was added, and samples were mixed by vigorous shaking. Samples were incubated on ice for 15 min and then centrifuged at 13,000 x g for 15min at 4°C. The aqueous phase was removed, combined with an equal amount of 70% ethanol, and applied to an RNeasy Mini Spin Column (Qiagen). RNA was eluted according to manufacturer’s instructions. RNA samples were analyzed for concentration
and purity on a Nanodrop 2000 spectrophotometer (Thermo Scientific). Complementary DNA (cDNA) was synthesized from RNA samples using QuantiTect Reverse Transcription kit (Qiagen) per the manufacturer’s instructions with 0.5 μg total RNA. Fourteen cycles of cDNA pre-amplification was performed according to manufacturer’s protocol using TaqMan PreAmp Master Mix (Applied Biosystems). Pre-Amplified cDNA was used for qPCR with the StepOne Plus Real Time PCR system (Applied Biosystems) in a 10 μl reaction for 40 cycles. Primers used were labeled with FAM probes include, Abca1 (Mm01350760_m1), ApoE (Mm00437573_m1), LXRa (Mm00443451_m1), PPARγ (Mm01184322_m1), GFAP (Mm01253033_m1), Iba1 (Mm00479862_g1), CD45 (Mm01293575_m1), App (Hs00245154_m1), Bace1 (Mm00478664_m1), Psen1 (Mm00501184_m1), Tnfa (Mm99999068_m1), Ccl2 (Mm00441242_m1), Il-1β (Mm01336189_m1), Nos2 (Mm01309902_m1), Cox2 (Mm01307329_m1), Ym1 (Mm00657889_m1), Fizz1 (Mm00445109_m1), Arg1 (Mm00475988_m1), Socs1 (Mm00782550_s1), Tgf-β (Mm01178820_m1) and GAPDH (4352339E-0904021) with a VIC probe from Applied Biosystems. Analysis of gene expression was performed using the comparative Ct method (ΔΔC_T), where the threshold cycle for the target genes was normalized to that of GAPDH (ΔC_T), and the mRNA expression fold change was calculated using the equation 2^−ΔΔC_T, where ΔΔC_T = ΔC_T test sample-ΔC_T calibrator sample.

Behavioral analysis (contextual fear conditioning) – In the training phase, mice were individually placed in the shock chamber to explore freely the environment for 2 min. Mice were exposed to the conditioned stimulus (CS: an 85dB sound at 2800Hz) for 30 sec. After 2 seconds, the unconditioned stimulus (US: 0.56mA) was delivered. After the
CS/US pairing, the mice were kept in the chamber for another 30 sec to measure immediate freezing response. This process was repeated 4 times. Retention tests were performed 24 hr later. Each mouse was returned to the same shock chamber for 5 min for contextual freezing measurements in the absence of tone and the number of freezes measured. Freezing behavior was monitored by automated tracking system (Coulbourn Instruments).
Results

PPARγ facilitates the degradation of soluble Aβ through an LXR/ApoE dependent pathway.

Previous work from our lab and others has demonstrated a role for LXR activation in stimulating amyloid clearance from the brain (Koldamova et al., 2005b, Riddell et al., 2007, Jiang et al., 2008, Donkin et al., 2010, Fitz et al., 2010). We sought to determine if activation of PPARγ, by using the synthetic agonist pioglitazone, would activate LXR pathways and induce clearance of Aβ though an ApoE dependent mechanism. Treatment of primary microglia and astrocytes with pioglitazone stimulated the degradation of soluble Aβ in a dose dependent manner (Figure 1A,D). Additionally, pioglitazone treatment of primary microglia and astrocytes resulted in a dose-dependent induction of the LXR target genes, ABCA1 and apoE (Figure 1B-F). To determine if degradation of soluble Aβ was dependent on the actions of PPARγ and LXRs, both microglia and astrocytes were pretreated with antagonists of these receptors (T0070907 and 22-S hydroxycholesterol (22HC) respectively). Antagonist treatment of microglia and astrocytes inhibited PPARγ-facilitated degradation of Aβ, suggesting that the activation of PPAR and LXR pathways is necessary for the intracellular degradation of Aβ in these cell types (Figure 2A, B).

ApoE has been shown to play a vital role in amyloid clearance and its expression is regulated by LXR activation (Jiang et al., 2008). Given the linkage of PPARγ to the LXR pathway, we sought to determine if ApoE played a critical role in PPARγ-mediated intracellular clearance of Aβ (Chawla et al., 2001). Microglia and astrocytes were
obtained from ApoE<sup>−/−</sup> pups and then treated with pioglitazone. Loss of apoE resulted in a significant impairment of Aβ degradation (Figure 2C, D) which was restored by the addition of exogenously supplied apoE or apolipoprotein A1 (apoA1) (Figure 2C,D). ApoA1 is an HDL-associated apolipoprotein which is lipidated by ABCA1, acts in the periphery in a similar fashion to ApoE, and has been shown to promote Aβ degradation (Koch et al., 2001, Smith et al., 2004, Koldamova et al., 2005a, Jiang et al., 2008). Exogenous apoE or apoA1 stimulated the clearance of Aβ peptides from the media. Addition of apoE or apoA1 to pioglitazone-treated microglia or astrocytes (wildtype or ApoE<sup>−/−</sup>) did not have additive or synergistic effects on Aβ degradation. These studies demonstrate that the effect of PPARγ agonists on Aβ degradation is dependent on the expression of ApoE and solely boosting levels of apolipoproteins is enough to facilitate Aβ clearance.

To determine the role of PPARγ in the expression of apoE and ABCA1, mice lacking PPARγ gene expression in astrocytes, as well as most cortical neurons, were obtained by crossing a PPARγ<sup>fl/fl</sup> mouse with a mouse expressing hGFAP-cre. The PPARγ conditional knockout (PPARγ CKO) display decreased levels of PPARγ mRNA that was reduced by over 70 percent in the cortex of these animals (data not shown). Six month old PPARγ CKO animals displayed over a 50% reduction in cortical levels of ABCA1 and ApoE, demonstrating the requirement for PPARγ for LXR action and the normal expression of its target genes apoE and ABCA1. This data documents a robust PPAR-LXR linkage in the brain which has not previously been recognized.

*PPARγ activation rapidly stimulates the clearance of Aβ in a mouse model of AD.*
To determine the effects of PPARγ activation during both early and more advanced stages of amyloid pathogenesis, 6 and 12 month old APPSwePSEN1ΔE9 (APP/PS1) mice were orally administered pioglitazone (80mg/kg/day) for 9 days. The APP/PS1 transgenic mouse model expresses the Swedish APP mutation (K595N/M596L) and mutant form of presenilin that lacks exon 9 that is expressed under the mouse prion promoter (Jankowsky et al., 2001). These animals begin depositing thioS+ plaques around 5 to 6 months of age. Pioglitazone treatment of 6 and 12 month animals resulted in a significant induction in cortical protein levels of both ABCA1 and ApoE (Figure 3A, B). Additionally, the assessment of LXR target genes by quantitative real-time PCR, showed statistically significant increase in levels of mRNA for Abca1, apoE and Lxrα. Pioglitazone-treated 12 month animals also showed an increase in levels of cortical Ppary mRNA (Figure 3A, F).

Immunohistochemical analysis of 6E10-immunoreactive plaques in cortical slices from these animals, revealed an approximate 40% reduction in total levels of deposited Aβ when compared to vehicle-treated APP/PS1 littermates (Figure 4A-C, E-G). Analysis of thioflavin S+ dense-core amyloid plaques paralleled these results, with about a 40% and 30% decrease in the number of thioS positive plaques in 6 and 12 month animals, respectively (Figure 4D, H). Remarkably, at 6 months of age pioglitazone treatment reduced soluble and insoluble levels of Aβ by approximately 50%. At 12 months of age, no significant change was seen in levels of soluble Aβ42, however, soluble levels of Aβ40 were reduced by 40% and insoluble levels of both Aβ40 and Aβ42 were reduced by 50% and 40%, respectively, following nine days of treatment (Figure 4I,J,L,M). Quantitative RT-PCR was carried out to verify that the changes seen in amyloid pathology in response
to PPARγ activation were due to increased proteolysis of the peptide and not to a change in levels of the amyloid precursor protein or components of the β and γ secretase. mRNA levels of App, Bace1 or Psen1 were not altered in response to drug treatment when compared to vehicle treated littermates (Figure 4K, N). Previous work has also suggested that PPAR activation may regulate the expression of key Aβ protease, insulin-degrading enzyme (IDE) and neprilysin (Du et al., 2009a, Kalinin et al., 2009). We monitored mRNA levels of IDE and neprilysin to determine if pioglitazone treatment enhanced expression of these proteases and observed no change (data not shown). These data suggest that the changes seen in amyloid deposition were indeed a result of Aβ catabolism and not due to alterations in APP processing.

*Pioglitazone suppresses glial activation and enhances amyloid clearance by these cells.*

The appearance of plaques in the AD brain is associated with a robust induction of astrogliosis and microgliosis. Microglia and astrocyte are found in intimate association with amyloid plaques in both the AD brain as well as in brains of AD mouse models (Itagaki et al., 1989, Wisniewski et al., 1992). The number of glial cells surrounding a plaque directly increase in proportion to the dimensions of the deposits (Stalder et al., 1999, Wegiel et al., 2001, Sasaki et al., 2002). Similarly, the induction of inflammatory mediators parallels amyloid deposition and the phenotypic activation of glial cells in the AD brain (Akiyama et al., 2000). Since PPARγ activation has been shown to result in potent anti-inflammatory actions and repress microglia-mediated inflammation, we wanted to examine the effects of pioglitazone treatment on glial
activation status and their association with amyloid plaques (Combs et al., 2001a, Hong and Tontonoz, 2008).

We first assessed association of astrocytes with amyloid plaques in the brains of both vehicle and pioglitazone treated 6 and 12 month APP/PS1 animals. Pioglitazone treatment significantly reduced GFAP expression in astrocytes surrounding amyloid plaques in transgenic animals at both 6 and 12 months of age (Figure 5A-L). Similarly, mRNA levels of Gfap was elevated in APP/PS1 animals, and was significantly reduced in both age groups with pioglitazone treatment (Figure 5N, O). In vehicle-treated animals, we observed GFAP immunoflourescence in close association with 6E10 positive plaques (Figure 5C, I). However, after pioglitazone treatment, astrocytes were seen diffusely surrounding Aβ deposits and very little contact was seen between GFAP positive cells and plaques (Figure 5F, L).

Surprisingly, in pioglitazone treated animals about 5-10% of astrocytes surrounding amyloid deposits in the cortex, displayed internalized Aβ, as seen in z-stack images obtained by confocal microscopy (Figure 5M). Internalization of Aβ was not observed in astrocytes from vehicle-treated APP/PS1 animals (data not shown). These data strongly support a role for astrocytes in Aβ clearance in vivo that is enhanced by PPARγ activation.

The association of Iba1+ microglial cells with amyloid plaques was reduced at both 6 and 12 months of age after pioglitazone treatment (Figure 6D-F, J-L). We found abundant microglia closely associated with plaque cores in vehicle-treated animals (Figure 6A-C, G-I). The mRNA levels for Iba1 were significantly lower in pioglitazone
treated animals compared to vehicle treated animals at 6 months of age and a trend
towards a reduction was seen at 12 months of age (Figure 6S, T). Remarkably, at 12
months of age, we found that most microglia in pioglitazone-treated APP/PS1 animals
exhibited internalized Aβ (Figure 7C, D). Microglia containing Aβ were occasionally
visualized in vehicle-treated animals, but were fewer in number and had small amounts of
Aβ when compared to those from the cortex of pioglitazone-treated animals (Figure 7A,
B). It is our qualitative assessment that up to 90% of the microglia in the cortex of drug-
treated animals internalized amyloid. Bolmont et al. have shown that microglia in the
AD brain are capable of taking up Aβ and delivering them to lysosomal compartments
(Bolmont et al., 2008). However, robust amyloid phagocytosis in response to drug
treatment, as seen here, has to our knowledge has never been observed before. Due to the
low numbers of Iba1+ cells in 6 month pioglitazone-treated animals it was difficult to
assess Aβ uptake by microglia in these animals. These data suggest that in addition to the
clearance of soluble forms of Aβ, pioglitazone treatment facilitated the phagocytic
removal of Aβ deposits by microglia and astrocytes.

We also evaluated the expression of CD45 by microglia, a tyrosine
phosphatase that plays an integral role in immune signaling. CD45 expression is elevated
in microglia in the AD brain and is associated with microglial activation (Masliah et al.,
1991, Wilcock et al., 2001). In 6 month old vehicle-treated animals, CD45
immunoreactivity was seen surrounding all 6E10 positive amyloid deposits (data not
shown). However, only approximately 50% of 6E10 positive deposits were associated
with CD45+ microglia in pioglitazone animals (data not shown). In 12 month animals
CD45+ microglia were associated with all amyloid deposits regardless of treatment
There was a trend for reduction in \( Cd45 \) mRNA levels in pioglitazone treated animals (Figure 6S, T). The reduction in levels of GFAP, CD45 and Iba1 in pioglitazone treated animals may be attributed to the role of PPAR\( \gamma \) in mediating suppression of an inflammatory response. Previous studies have documented a role for PPAR\( \gamma \) in polarizing peripheral macrophages to an alternative activation phenotype that is anti-inflammatory and engages the phagocytic activity of the cells. Thus, we next examined markers of alternative activation which have been known to exert an anti-inflammatory phenotype and promote tissue repair and phagocytosis (Mosser and Edwards, 2008, Chawla, 2010). At 6 months of age \( Fizz1 \) levels were significantly reduced in vehicle and pioglitazone-treated APP/PS1 animals. Six month old APP/PS1 animals showed no differences in levels of \( Yml \) or \( Arg1 \) when compared to WT animals. Pioglitazone, however, dramatically increased levels of the alternative activation marker, \( Yml \) mRNA in APP/PS1 animals (Figure 7E). At 12 months of age pioglitazone treatment dramatically increased the expression of all M2a markers assayed, \( Fizz1, Yml \) and \( Arg1 \). \( Fizz1 \) levels remained reduced in 12 month old transgenic animals (Figure 7F). Brain levels of \( Il-1\beta \) and \( Tnf\alpha \) were also reduced in these animals, verifying the anti-inflammatory effects of PPAR\( \gamma \) activation (data not shown). Additionally, we monitored an increase in levels of the M2c marker \( Tgf-\beta \) in 12 but not 6 month old APP/PS1 mice. TGF-\( \beta \) is associated with an “acquired deactivation” or “M2c” state that is associated with a suppression of the innate immune system and increased phagocytic capacity that is normally needed to remove cellular debris and apoptotic cells (Van Ginderachter et al., 2006, Mandrekar-Colucci and Landreth, 2010). These data suggest a conversion of microglial phenotype in APP/PS1 animals, from the classical M1 to alternative M2 state.
following PPARγ activation, promoting the phagocytic removal of amyloid deposits by microglial cells.

*PPARγ activation ameliorates Aβ-related behavioral deficits.*

Finally, we examined the effects of PPARγ activation on associative memory in APP/PS1 animals using a controlled fear conditioning assay. Twelve month old transgenic animals were treated orally for 9 days and trained and tested for contextual memory following a contextual fear conditioning protocol. Animals were trained for four consecutive training sessions and all groups showed signs of learning (Figure 8B). However, vehicle treated APP/PS1 animals showed significant impairments in memory retention as evidenced by the low number of freezes in the cued environment (Figure 8A). Treatment of APP/PS1 mice with pioglitazone resulted in significant behavioral improvement that was not different from wildtype animals. Additionally, pioglitazone treatment also reversed non-cognitive behavioral deficits seen in APP/PS1 animal and restored distance and speed traveled to levels comparable to wildtype animals in an open field (Lalonde et al., 2005). These results demonstrate that PPARγ activation results in the rapid improvement of memory in an AD mouse mode.
Discussion

The roles of PPARγ in the brain and its potential as a therapeutic target for the treatment of AD have generated substantial controversy. The primary aim of the present study was to define the cellular mechanisms through which it acts in AD pathogenesis and demonstrate its activation results in very rapid and robust amelioration of the principal pathologic features and functional impairments in an animal model of AD. Much of the controversy over the action of PPARγ agonists arises from the poor blood brain barrier (BBB) permeability of its agonists, both in mice and humans. We demonstrate that, once activated in the brain, PPARγ acts coordinately through two parallel pathways resulting in the rapid metabolism of Aβ and reversal of cognitive deficits in an AD mouse model.

The thiazolidinedione (TZD) agonists of PPARγ, pioglitazone (Actos™) and rosiglitazone (Avandia™) have been shown to exert potent anti-inflammatory actions and are efficacious in a number of CNS disease models. Several studies have utilized TZDs to determine their therapeutic potential for AD, but have yielded conflicting results in animal models of AD. Rosiglitazone is known to have very poor BBB penetrance and is actively effluxed from the brain by p-glycoprotein (Hemauer et al., 2010). Pioglitazone has been shown to penetrate the BBB, but poorly (Maeshiba et al., 1997). Low doses of pioglitazone have been shown to effectively lower only soluble levels of Aβ₄₂ and improve behavior in some instances (Yan et al., 2003, Heneka et al., 2005, Nicolakakis et al., 2008). Rosiglitazone treatment has also been demonstrated to have beneficial effects on AD pathology and cognitive impairments, in spite of its poor BBB permeability, however, the mechanisms of actions of this agonist remains unclear.
ApoE is the principal genetic risk factor for sporadic forms of AD, and its precise roles in AD pathophysiology has remained enigmatic. We have previously demonstrated that one critical function of apoE is to facilitate the degradation of soluble species of Aβ (Jiang et al., 2008). The ability of apoE to enhance the degradation of Aβ is reliant on the functions of ABCA1 and its ability to lipidate apoE containing HDL particles. We have shown that activation of LXRα, facilitates the proteolytic degradation of Aβ in an apoE-dependent mechanism through induction of its target genes Abca1 and apoE. Importantly, PPARγ induces LXRα expression and we hypothesized that PPARγ acts through an LXR pathway to reduce amyloid burden in an AD mouse model (Chawla et al., 2001).

Due to the poor BBB permeability of pioglitazone, we administered a high dose of pioglitazone (80/mg/kg/day). Moreover, unlike previous studies, we have employed a very short treatment interval (9 days), owing to the expeditious induction of receptor-mediated gene expression. Indeed, our results demonstrate that PPARγ activation not only it also induces the expression of the LXR target genes apoE and ABCA1 but also rapidly facilitates the degradation of Aβ (Figure 1, 4) both in vitro (Figure 1 ) and in vivo (Figure 3) in an AD mouse model. One of the principal outcomes of this study is the demonstration of a strong link between PPARγ activation, induction of LXR targets and amyloid clearance in the brain, demonstrating that a robust PPAR-LXR-ApoE pathway in the CNS and can be engaged to facilitate the clearance of Aβ.

Another striking finding in this study was the extraordinary ability of microglial cells to phagocytically take up and remove amyloid deposits from the AD brain (Figure 7). The heterogeneity of microglial phenotype in healthy and diseased states is just recently beginning to be understood (Martinez et al., 2009, Mandrekar-Colucci and
Landreth, 2010). It is now known that macrophages/microglia are capable of exhibiting a spectrum of phenotypic activations states which dictate their activities in the brain. Nuclear receptors have only recently been appreciated to play an crucial role in the phenotypic change of microglia from a “classically” activated to an “alternatively” activated state (Odegaard et al., 2007, Zelcer et al., 2007, Odegaard et al., 2008, Kalinin et al., 2009). Activation of these nuclear receptors not only suppresses NFκB-mediated inflammatory responses but also has shown to activate the phagocytic machinery of microglia/macrophages (Landreth and Heneka, 2001, Zelcer et al., 2007, Mukundan et al., 2009). In the AD brain, microglia fail to effectively phagocytose Aβ and are also impaired in their ability to degrade Aβ fibrils (Frackowiak et al., 1992, Chung et al., 1999, Rogers et al., 2002). Thus, modulating microglial activation status may be the key to regaining their phagocytic functionality in the CNS (Majumdar et al., 2007).

Microglia in pioglitazone-treated animals were more efficient at ingesting Aβ fibrils when compared to microglia from vehicle-treated animals. Additionally, pioglitazone-treated animals displayed an induction of the alternative activation genes *Ym1*, *Fizz1* and *Arg1* and a parallel reduction in *TNFa* and *Il-1β* levels in the cortex, demonstrating a switch in microglial activation from a “classical” to “alternative” activation state (Figure 7). It is reasonable to propose that PPARγ induced polarization of microglial cells to a M2 “alternative” state stimulated the phagocytosis and clearance of Aβ fibrils.

Microglia are the principal immune effector cells in the CNS and are the only professional phagocyte in the CNS. Thus, microglia have been the main focus of many studies investigating amyloid clearance. An important finding in this study was the demonstration of the ability of astrocytes to efficiently take up and degrade Aβ *in vivo*
Astrocytes greatly outnumber microglia in the CNS, and their ability to remove even modest amounts of Aβ in the brain may have a significant impact on amyloid clearance in the brain. Indeed, astrocytes were more sensitive to pioglitazone treatment in vitro and were able to facilitate degradation of soluble Aβ peptides at a lower dose, compared to microglial cells (Figure 1). Additionally, amyloid-laden astrocytes were only found in the cortex of pioglitazone treated animals and not in vehicle treated APP/PS1 mice (Figure 5). Several studies have shown that astrocytes are capable of taking up amyloid peptides in vitro and that this ability is restricted to adult and not neonatal astrocytes (al-Ali and al-Hussain, 1996, DeWitt et al., 1998, Wyss-Coray et al., 2003, Pihlaja et al., 2008). Studies have also revealed an association of Aβ peptides and astrocytes in vivo in humans or in mouse models of AD (Funato et al., 1998, Matsunaga et al., 2003, Nagele et al., 2003). However, due to the imaging procedures utilized in these studies, it is difficult to determine if Aβ is internalized within the astrocytic cell. In this study, we have utilized confocal z-stack images that demonstrate internalization of Aβ peptides within astrocytes surrounding amyloid plaques. To our knowledge, our study represents the first in vivo documentation of astrocytic uptake of Aβ peptides as a result of drug treatment.

Interestingly, a paper published by Koistinaho et al, described that the ability of astrocytes to internalize and degrade Aβ deposits in an ApoE-dependent manner using an ex vivo culture system (Koistinaho et al., 2004). It is important to note that in our experimental paradigm, PPARγ activation elevated brain apoE levels (Figure 3) and this may facilitate the recognition and uptake of Aβ fibrils. This study strongly supports the view that PPARγ activation initiates amyloid clearance pathways in astrocytes as well as
microglia and suggests a reevaluation for the role of astrocytes in amyloid clearance in AD.

Owing to the success of PPARγ activation in mouse models of AD, small clinical trials evaluated the effects of receptor activation in patients with mild to moderate AD. Treatment of these patients with pioglitazone improved memory and cognition (Hanyu et al., 2009, Sato et al., 2009). A larger phase II clinical trial showed improvements in memory retention and attention with the treatment of rosiglitazone (6 months) in patients that did not possess an ApoE4 allele (Risner et al., 2006). Unfortunately, phase III clinical trials, utilizing rosiglitazone, failed to show efficacy for the treatment of AD (Gold et al., 2010). Importantly, these trials administered rosiglitazone at dosages that were less than 10% of that needed to see beneficial effects on AD pathophysiology in rodent models of the disease. Furthermore, the drug that was utilized, rosiglitazone, has poor BBB penetrance, and is known substrate for p-glycoprotein mediated efflux, the activity of which is known to be upregulated in response to inflammatory cytokines, further limiting the exposure of rosiglitazone to the CNS (Bauer et al., 2007, Gendelman, 2007, Hemauer et al., 2010). Due to poor design of the phase III clinical trials, it is impossible to assess the efficacy of these compounds in the treatment of AD.

In this study we have demonstrated the rapid effects of PPARγ activation on amyloid clearance, microglial polarization and the reversal of cognitive deficits associated with AD. We have shown a mechanistic linkage between the PPARγ and LXR pathways, which has never before been documented in the brain, and results to the production of lipidated ApoE particle, facilitating the degradation of soluble Aβ species.
We have also reported that PPARγ activation changes the inflammatory milieu of the brain by phenotypically polarizing microglia to an alternative, M2 phenotype, allowing them to phagocytically remove amyloid deposits. These data suggest that PPARγ agonists remain of therapeutic utility for the treatment of AD.
**Figure 1: Activation of PPARγ facilitates the expression of LXR target genes and promotes the degradation of Aβ.** Primary microglia (A) or astrocytes (D) were isolated from wildtype mice and pretreated with either DMSO (Ctrl) or increasing concentrations of pioglitazone (Pio) for 24 hrs. The cells were then incubated with 2μg/ml of Aβ in the presence of DMSO or the indicated dose of pioglitazone for 24 hrs. Intracellular Aβ levels were quantified using ELISA for Aβ_{42} and normalized to total protein (mean ±SEM, ***p < 0.001). Primary microglia (B, C) or astrocytes (E, F) were treated with DMSO or increasing doses of Pio for 24 hrs. Cellular lysates were subjected to SDS-PAGE and western blotted for ABCA1, ApoE or actin (mean ± SEM, Student’s T test *p< 0.05, **p< 0.01, n≥3). These data are represented as a percentage of DMSO-treated control samples.
A

All Relative Percentage (Normalized to Ctrl Treatment)

Pio (nM) Ctrl 25nM 50nm 100nM

B

ABCA1
Actin

C

ApoE
Actin

D

All Relative Percentage (Normalized to Ctrl Treatment)

Pio (nM) Ctrl 5nM 25nM 50nM

E

ABCA1
Actin

F

ApoE
Actin
Figure 2: PPARγ mediated degradation of Aβ is dependent on induction of ApoE through the stimulation of LXR and PPAR pathways. Primary wildtype microglia (A) or astrocytes (B) were treated for 24 hrs with pioglitazone or DMSO followed by the addition of soluble Aβ42 for 24 hrs. The cells were pre-treated with competitive agonists for PPARγ (T0070907, 10nM) or LXR (22-S hydroxycholesterol; 22HC, 10μM) for 2 hours. Intracellular Aβ was measured by ELISA. ApoE knockout microglia (C) or astrocytes (D) or wildtype microglia (E) or astrocytes (F) were pretreated for 24 hours with DMSO or drug, followed by the addition of soluble Aβ42 and exogenously supplied ApoE (1μg/ml) or ApoA1 (2μg/ml). Remaining intracellular Aβ was measured using ELISA. These data are represented as a percentage of DMSO treated control samples (mean ± SEM, Student’s T test, *p<0.05, **p<0.01, ***p<0.001, n≥3). Cortical homogenates obtained from PPARγ conditional knockout animals were analyzed by western blot analysis for ABCA1, ApoE and actin (G) and quantified (H). (mean ±SEM, Student’s T test, *p<0.05, ***p<0.001, n≤6 animals per genotype).
Figure 3: PPARγ activation drives the expression of LXR target genes in vivo. Six (A, C) and twelve (B, D) month old APP/PS1 mice were gavaged orally with 80mg/kg/day of pioglitazone or vehicle (Veh) or wildtype (WT) animals were gavaged with vehicle for 9 days. Cortical homogenates were then analyzed by western blot for levels of ABCA1, ApoE and Actin (A, B) and quantified (C, D). (mean ± SEM, *p<0.05, **p<0.01, n≥6 animals/treatment). Quantitative PCR results for LXR target genes from cortices of 6 (E) or 12 (F) month old APP/PS1 animals treated with pioglitazone or vehicle for 9 days. (mean ± SEM, Student’s T test, *p< 0.05, **p< 0.01, n≤13 animals/group). Fold change is reported to vehicle treated WT animals.
4: Pioglitazone treatment ameliorates amyloid deposition in APP/PS1 mice.

APP/PS1 mice were gavaged for 9 days with pioglitazone (80mg/kg/day) or vehicle (water). Plaque pathology was evaluated in the cortex of vehicle or pioglitazone treated 6 (A-D, I-K) or 12 month (E-H, L-N) animals by staining with 6E10 (anti-Aβ antibody) (A,B,E,F) or thioflavin S. Quantification of 6E10 % area (C,D) and number of ThioS positive plaques (D, H). Soluble and insoluble levels of Aβ40 and Aβ42 were measured by ELISA in 6 (I, J) and 12 month animals (L, M). Real time quantification of mRNA levels of APP and the APP proteases BACE and PSEN1 were measured in 6 (K) and 12 (N) animals to determine if pio treatment altered the expression of enzymes involved in APP processing. (mean ± SEM, Student’s T test, *p<0.05, **p<0.01, ***p<0.001, n≥6 animals/group).
Figure 5: PPARγ suppresses astrocytosis in APP/PS1 animals. GFAP and 6E10 immunohistochemistry on coronal sections from 6 (A-F) or 12 (G-L) month old APP/PS1 animals treated with pioglitazone (D-F, J-L) of vehicle (A-C, G-I) for 9 days. (Magnification is 20x). Representative image of astrocytes from the cortex of 12 month pioglitazone treated animal (M) (Red:6E10, Green:GFAP, Blue:Dapi, 100x magnification. Quantitative real time PCR in vehicle treated WT or APP/PS1 animals or pioglitazone treated APP/PS1 animals for GFAP (N, O). (mean ± SEM, Student’s T test, *p<0.05, **p<0.01, ***p<0.001, n≤13 animals/group).
Figure 6: Pioglitazone suppresses microglial activation surrounding amyloid plaques in APP/PS1 animals. Cortical Iba1 and 6E10 immunochemistry on coronal sections from 6 (A-F) or 12 (G-L) month old vehicle (A-C, G-I) or pioglitazone (D-F, J-L) treated APP/PS1 animals. Cortical CD45 and 6E10 staining in 12 month old vehicle (M-O) or pioglitazone (P-R) treated animals. Real-time PCR quantification of transcript levels of Iba1 or CD45 in 6 (S) and 12 (T) month old pio or vehicle treated APP/PS1 animals. (mean ± SEM, Student’s T test, *p<0.05, n≤13 animals/group).
Figure 7: Pioglitazone treatment enhances microglial phagocytosis of Aβ.
Representative images from vehicle (A, B) or pioglitazone (C, D) treated 12 month old APP/PS1 animals. (A) and (C) represent projection images of zStacks (B, D). (Green: Iba1, Red:6E10, Blue:DAPI, images are acquired using a Zeiss LSM 510 confocal microscope, magnification is 100x).
Figure 8: PPARγ activation reverses cognitive deficits in 12 month old APP/PS1 animals. Contextual fear memory assessment was examined in 12 month old vehicle or pioglitazone (80mg/kg/day) treated APP/PS1 animals (A). (n= 7-10 animals/group). The number of freezes were shown as a function of training periods for contextual fear condition (B). Measurement of distance traveled (C) and mean speed (D) in vehicle treated WT and APP/PS1 and pioglitazone treated APP/PS1 animals.
Chapter 4

Discussion
The amyloid hypothesis, first described by John Hardy in 1991, suggests that the principal mechanisms underlying Alzheimer’s disease (AD) are those that promoting the accumulation of the amyloid beta (Aβ) protein in the brain. This could occur through a variety of processes including dysfunction in APP metabolism or impairments in Aβ clearance mechanisms. The gradual accumulation of this peptide within the brain, over long periods of time, results in large amounts of Aβ that are neurotoxic and affect neurons in the surrounding environment (Tanzi and Bertram, 2005). This buildup of Aβ has been shown to disrupt synaptic function, impair mitochondrial metabolism, induce apoptosis, and impair insulin signaling. One of the most important effects of the presence of fibrillar forms of Aβ is that it phenotypically activates microglial cells leading to the production of pro-inflammatory cytokines that are detrimental to the surrounding brain tissue (Bamberger and Landreth, 2001, Xie et al., 2002, Caricasole et al., 2003, Lustbader et al., 2004).

The primary focus of this thesis has been to elucidate mechanisms of soluble Aβ uptake that promote clearance of this peptide from the brain. We show that microglial cells take up soluble forms of Aβ peptide through fluid-phase pinocytosis. This mechanism is distinct from the phagocytosis of amyloid fibrils (Mandrekar et al., 2009). Furthermore, we have shown that activation of the nuclear receptor peroxisome proliferator activated receptor-γ (PPARγ) promotes the proteolytic degradation of soluble Aβ by both microglia and astrocytes and facilitates the clearance of amyloid pathology in a mouse model of AD, ameliorating AD-related cognitive deficits. One of the surprising findings that emerged from this study was that activation of PPARγ, using pioglitazone, was the enhanced clearance of dense-core amyloid deposits in the cortex of an AD mouse.
model. While PPAR-mediated activation of LXR pathways leading to the induction of ApoE expression explains the clearance of soluble Aβ peptides, it does not explain the reduction in insoluble deposits. Further analysis revealed that PPARγ activation played a role in microglial polarization in the brain, a mechanism that potentially explains the re-engagement of the phagocytic machinery and the subsequent ability of microglial cells to phagocytose and degrade Aβ fibrils.

The second interesting finding in these studies was the ability of pioglitazone treatment to promote astrocytic uptake and clearance of Aβ, a process originally thought to take place in only in microglial cells. While astrocytes have been documented to take up amyloid peptides, we provide the first evidence documenting a drug treatment that facilitates the clearance of Aβ by astrocytes in an in vivo model of AD (Funato et al., 1998, Matsunaga et al., 2003, Nagele et al., 2003, Koistinaho et al., 2004, Pihlaja et al., 2008).

The work documented in this thesis provides an in-depth understanding of the role of the nuclear receptor PPARγ in AD pathophysiology and we have raised a number of new questions that remain to be answered and will discussed in detail in this section.

Microglia M2 (alternative) polarization by PPARγ activation

*PPARγ induces alternative activation of microglia in the cortex of APP/PS1 animals*

A central question that remains unanswered in how PPARγ activation promotes the uptake and degradation of fibrillar Aβ deposits. We have shown that treatment of
APP/PS1 animals with pioglitazone for 9 days rapidly ameliorates Aβ pathology in the cortex of these animals. The attenuation of amyloid pathology correlated with an approximate 40% reduction in brain levels of insoluble Aβ40 and Aβ42 and a 40% reduction in the number of thioS+ plaques in the cortex. This dramatic reduction in Aβ levels is consistent and found in pioglitazone treated animals that are 6 or 12 months of age. We have also documented a change in microglial polarization in response to pioglitazone treatment in APP/PS1 animals. After 9 days of drug treatment of 12 month old animals showed significant increases in levels of the alternative activation markers, Ym1, Fizz1 and Arg1. These animals also display a decrease in levels of Il-1β and Tnfα that are induced by Aβ deposition (Figure 1).Vehicle-treated transgenic animals, however, did not display an alteration in levels of alternative activation genes when compared to non-treated WT animals. This result conflicts with findings from Colton et al who see an increase in Arg1 and Ym1 levels in 17 month old Tg-2576 animals and Jimenez et al. who document a significant increase in YM1, but not Arg1 levels, in 6 and 12 month old APPxPS1 animals (Colton et al., 2006, Jimenez et al., 2008). These discrepancies may arise as an artifact of the mouse model utilized in these studies, as well as the kinetics of Aβ deposition at the various ages observed.

While the exact role of the protein products of these alternative activation genes remains unknown, they are thought to be immunosuppressive and play an integral role in tissue repair. The YM1 protein, codes for a mammalian lectin that has been reported to bind heparin/heparin sulfate and saccharides on the surface of cells (Raes et al., 2002). It is been postulated that this interaction may reduce heparin sulfate degradation at sites of injury and thus protect the extracellular matrix at sites of damage (Hung et al., 2002).
Although there is no human homolog of the YM1 gene, the human AD brain does express two other closely related chitinase genes, CHI3L1 and CHI3L2 that may play similar roles to YM1 (Ling and Recklies, 2004, Colton et al., 2006). FIZZ1 is a resistin-like molecule and has been shown to play a role in insulin resistance, angiogenesis and the inhibition of apoptosis (Holcomb et al., 2000, Sandler et al., 2003). Arginase 1 (Arg1) is probably the most widely studied of the alternative activation genes. Arg1 utilizes arginine as a substrate to generate proline and polyamines, necessary for tissue repair, and diverts arginine metabolism away from the production of nitric oxide (Williams, 1997, Wu and Morris, 1998, Hesse et al., 2001).

Pioglitazone treatment of 12 month old APP/PS1 animals positively affected the inflammatory environment of the brain. Levels the pro-inflammatory cytokines IL-1β and TNFα were reduced and genes associated with tissue repair and dampening of the inflammatory response were upregulated. One surprising result was that levels of Nos2 were unaffected with drug treatment (Figure 1). Since inducible Nos and arginase enzymatic activities are dependent on arginine levels, and levels of Arg1 are seen elevated with pioglitazone treatment, we expected a dramatic reduction in levels of Nos2 (Wu and Morris, 1998). However, levels of Nos2 were not elevated in vehicle treated transgenic animals and these results are consistent with findings in the Tg2576 and the PS1xAPP mouse model of AD (Colton et al., 2006, Jimenez et al., 2008). Jimenez et al did not observe significant elevations in COX2 or Nos2 levels in APPxPS1 mice until 18 months of age (Jimenez et al., 2008).

Currently, very few antibodies are available to monitor alternative activation of microglia by immunohistochemistry. Based on our results, it would be interesting to see
the phenotypic characteristics of different populations of microglial cells, specifically those surrounding amyloid plaques compared to those dispersed in the cortex, and to ascertain how these cells respond to pioglitazone treatment. Based on our qRT-PCR data and confocal images, we expect that pioglitazone treatment will polarize plaque-associated microglia and stimulate their expression of the M2 markers YM1, Arg1 and Fizz1, while downregulating the expression of inflammatory cytokines. It is unclear, however, how the phenotypic state will be altered in microglia that are not in the vicinity of amyloid deposits. Studies by Gordon and colleagues, suggest that the polarization of macrophages in response to a stimulus is not only dependent on the stimulus itself, but also the prior activation state of that cell (Varin et al., 2010). Since the parenchymal microglia are not stimulated by plaque association, it would be interesting to characterize their initial activation state and how it is affected by PPARγ activation.

While these phenotypic changes in activation states have been attributed to microglial cells, which are thought to be the main contributors to the inflammatory milieu seen in AD, it is possible that other cell types (neurons, astrocytes, oligodendrocytes) in the CNS have the ability to express markers for alternative activation. Originally, it was thought that since microglial cells were the mediators of the innate immune system in the brain they were the sole expressers of pattern recognition receptors. However, recent evaluation of this theory has shown that in addition to microglia astrocytes can also express toll-like receptors (Falsig et al., 2008, Gorina et al., 2011, Henn et al., 2011). Thus, it is important to assess the contribution of other cell types to the expression of genes that are associated with macrophage/microglial polarization in the AD brain induced by PPARγ activation. Initially, it would be important to see if other cell types in
the brain can express the alternative activation markers. This can be achieved by using primary cultures, stimulating them with M1 or M2 polarizing agents and monitoring the expression of M1 and M2 markers by qRT-PCR. If these experiments yield positive results then it would be interesting to see the effects of PPARγ activation on modulating the phenotypic polarization of these cell types both in vitro through qRT-PCR and in vivo by utilizing immunohistochemistry.

Effects of M2 polarization by PPARγ activation on phagocytosis

In addition to an increase in the prototypical alternative activation genes, we also see an increase in levels of TGFβ, an M2c marker, following PPARγ activation. The M2c state is termed “acquired deactivation” and is associated with increased phagocytosis, usually of apoptotic bodies. Recent studies have shown that activation of both PPARδ and LXR can stimulate the deactivation of microglia/macrophages and induce engulfment of apoptotic cells (Mukundan et al., 2009, N et al., 2009). Together these finding suggest that PPARγ activation switches the phenotypic activation state of the microglia in the brain from a classical state to an alternative activation state. The role of PPARγ in macrophage polarization has been documented in a number of studies (Bouhlel et al., 2007, Charo, 2007, Odegaard et al., 2007, Odegaard et al., 2008, Mukundan et al., 2009). A putative PPRE has even been found in the distal enhancer region of the Arg1 gene (Odegaard et al., 2007). Additionally, they exert anti-inflammatory actions thought the inhibition of NFκB promoters (Pascual et al., 2005, Straus and Glass, 2007). The change in microglial activation we observed is paralleled with reductions in insoluble levels of Aβ and the appearance of microglial cells
containing Aβ. The question remains whether this change in microglial activation status is directly responsible for the phagocytic uptake of Aβ by microglial cells.

Indeed, stimulation of microglial cells with anti-inflammatory cytokines, IL-4, IL-13 and TGF-β or LXR agonists has been shown to relieve the suppression of pro-inflammatory cytokines of phagocytosis by microglial cells (Koenigsknecht-Talboo and Landreth, 2005, Zelcer et al., 2007). PPARγ stimulation or exposure of microglial cells to anti-inflammatory cytokines alone does not increase their phagocytic capacity (data not shown) (Koenigsknecht-Talboo and Landreth, 2005). However, it is unknown whether PPARγ can rescue Aβ stimulated phagocytosis in microglia exposed to inflammatory cytokines. This question can be easily addressed in an in vitro culture system using a phagocytosis assay we have established in our laboratory. Primary microglial cells obtained from wildtype P0-P3 B6 pups will be treated on day one with inflammatory cytokines (INFγ, TNFα, IL-1β or LPS) for 18 hours. The following 18 hours microglia will be treated with the PPARγ agonist, pioglitazone, in the presence of inflammatory cytokines. On day three microglia will be stimulated for 30 min in the presence of the inflammatory cytokines (+/- pioglitazone) with fibrillar Aβ_{42} followed by a 30 incubation with 1μm fluorescent microspheres. The fraction of phagocytically active cells can then be determined by FACS analysis. Stimulation of microglia with inflammatory cytokines should suppress Aβ-induced phagocytosis of 1 micron fluorescent beads. If PPARγ agonist treatment polarizes microglia from an M1 (classical) to M2 (alternative) activation state and affects the phagocytic capacity of the cells, we would expect that PPARγ agonist treatment to relieve the phagocytic suppression induced by pro-inflammatory cytokine exposure and an increase in the
number of cells that have internalized phagocytic beads. Additionally, by using FACS analysis we can also calculate the number of beads taken up by each microglia to determine if the phagocytic capacity of each cell is increased with pioglitazone treatment.

Additionally, it would be very interesting to see, in vivo, if the phagocytic cells observed in the pioglitazone treated APP/PS1 animals, were in fact M2 polarized. To determine this, 12 month old APP/PS1 animals would be treated for 9 days with either vehicle (water) or pioglitazone (80/mg/kg/day). Prior to initiation of treatment animals would be injected intraperitoneally with methoxy-XO4, a congo red derivative, that infiltrates the brain and labels amyloid plaques. This will allow for labeling of the amyloid plaques before treatment so that phagocytic uptake of pre-deposited plaques can be monitored. After treatment completion animals will be sacrificed and brain microglia will be isolated (using CD11b antibody) and sorted for cells containing methoxy-XO4 labeled amyloid peptide by using FACS analysis. These cells will then be pooled and assessed for markers of M1 and M2 activation states. We suspect that microglia isolated from pioglitazone treated animals that contain Aβ will robustly express genes of alternative activation, while cells that do not contain Aβ will express phenotypic markers of M1 activation states. Similarly, we expect to see very few microglia in vehicle treated animals that contain significant amounts of Aβ. We expect that the activation of these cells will exhibit a M1 polarized phenotype.

*M2 Polarization and Lysosomal Acidification*

In the AD brain, amyloid deposits are associated with an abundance of microglial cells. While these cells are found in close proximity and intimately associated with the
amyloid deposits they are unable to take up and clear fibrillar Aβ species. APP/PS1 animals treated with pioglitazone display masses of amyloid-laden microglial cells. Additionally, decreases in insoluble Aβ and thioS positive plaques suggest that microglia not only take Aβ but also efficiently degrade the peptide. This is quite interesting, because in vitro data has shown that while microglia can take up fibrillar Aβ and deliver the peptides to the lysosome they are unable to degrade the peptide and have been shown to retain them for up to seven days in vitro (Paresce et al., 1997a, Chung et al., 1999). A compelling study published recently by Majumdar et al found that the key to amyloid degradation lies in acidification of lysosomes. They evaluated the lysosomal pH and protease content in both macrophages (which can degrade Aβ efficiently) and primary microglial cells (Majumdar et al., 2008). They found that while microglial cells contained higher levels of proteases, their lysosomes were less acidic (pH 6.0) when compared to macrophages (pH 5.0). This difference in pH resulted in a reduction of lysosomal enzymatic activity in microglial cells, possibly explaining their inability of these cells to degrade Aβ fibrils. More interestingly, stimulation of microglial cells with a M2 polarizing agent, M-CSF, resulted in the acidification of microglial lysosomes and a rescue of their ability to degrade Aβ (Verreck et al., 2004, Majumdar et al., 2007, Brocheriou et al., 2011). This study suggests that utilizing agents that modulate lysosomal pH levels in microglial cells may be of therapeutic utility for AD treatment. Similarly, it suggests M2 polarizing agents may play a role in lysosomal acidification.

We have shown that PPARγ activation is an active M2 polarizing agent, and that treatment of APP/PS1 seems to re-engage the phagocytic machinery of microglial cells and facilitate the phagocytosis of fibrillar Aβ deposits. PPARγ mediated amyloid
clearance may occur by modulating microglial pH levels. This could be tested by employing dextran conjugated to pH sensitive and insensitive dyes, in method similar to that carried out by Majumdar and colleagues. To mimic the AD brain, primary microglial cells will be primed using a M1 stimulus such as inflammatory cytokines for a minimum of 24 hours before the addition of the M2 stimulus, pioglitazone, for an additional 24 hours. Cells will then be loaded with dextran labeled with pH-sensitive fluorescein and pH-insensitive rhodamine for 30 min after which dextran will be washed from the media. The lysosomal pH will then be monitored using real time imaging. Images will be taken every 30 min for 2 hours and lysosomal pH will be evaluated based on the ratio of endocytosed fluorescein to rhodamine labeled dextran. This analysis can also be carried out using FACS analysis of the cells, however, this method would not allow for a time-dependent analysis of changes in lysosomal pH. Non-treated cells will be used as baseline controls. Similarly, M2 polarized cells will be compared to cells that have only received a M1 stimulus. Additionally, this experiment could also be carried out using double labeled (with pH sensitive and insensitive dyes) Aβ fibrils. This would not only allow us to monitor lysosomal pH but also evaluate stimulus-dependent degradation of amyloid fibrils in microglia. It is unclear, however, how fluorescently labeling Aβ fibrils affects their functionality and this could be a potential flaw in using fluorescently labeled Aβ fibrils. Based on Majumdar’s results we anticipate pioglitazone treatment to induce changes in lysosomal pH, facilitating their acidification and stimulating pH-dependent enzymatic activity of lysosomal proteases resulting in Aβ degradation (Majumdar et al., 2007). Similarly, since pro-inflammatory cytokines repress
phagocytosis, it would be interesting to see how treatment of microglial cells with M1 polarizing agents affects lysosomal pH.

**PPARγ mediated polarization of resident microglia vs infiltrating monocytes/macrophages**

The role of peripheral monocytes/macrophages in the AD brain has been a topic of much controversy. The idea that bone-marrow derived microglial cells from the periphery could invade the AD brain, home to amyloid plaques and clear Aβ more efficiently than their resident counterparts was first introduced by Simard and Rivest (Simard and Rivest, 2006, Majumdar et al., 2007). Later studies challenged this idea demonstrating that the techniques utilized in these experiments compromised the blood brain barrier (BBB) and facilitated the migration of cells into the brain that would not have been able to cross the BBB otherwise (Ajami et al., 2007, Mildner et al., 2007b). Studies employing a parabiotic mouse system or injections of bone marrow derived cells into the circulatory system, have shown that peripherally derived microglia/macrophages cross the BBB, enter the CNS and migrate to areas of damage (Tzeng and Wu, 1999, Massengale et al., 2005, Lebson et al., 2010). In the AD brain, these peripherally derived cells, which are defined as CX3CR1lo/Ly6Cahi/CCR2+, and are attracted and migrate to amyloid deposits. This migration is in response to the high levels of chemokine (C-C motif) ligand 2 (CCL2), that are secreted by plaque-associated microglia and astrocytes, and act as a ligand for CCR2 (Prinz and Priller, 2010). Indeed, bone marrow cells derived from CCR2−/− animals are unable to infiltrate the AD brain (El Khoury et al., 2007).
Numerous studies have shown the importance of PPARγ for optimal expression of alternative activation markers (Bouhlel et al., 2007, Charo, 2007, Odegaard et al., 2007). One study of particular interest to our findings was conducted by Bouhlel et al. and demonstrated that PPARγ activation was a necessary event in priming peripheral monocytes to a M2 phenotype. Significantly, PPARγ activation did not affect the expression of M2 markers in resting or M1 polarized macrophages, suggesting that once the cell expresses M1 markers, PPARγ no longer has effects on polarization of the cell.

It is unknown, in our paradigm, whether the change in the general activation status of the brain in response to pioglitazone treatment is due to a change in phenotype of CNS-resident plaque-associated microglia from classical to alternative activation phenotypes, or whether it is due to an influx of peripherally-derived M2 primed monocytes, which have now entered the brain and differentiate into a microglial “type” cell.

At this moment, due to the lack of markers that differentiate between resident microglia and peripherally derived macrophages, we do not have the ability adequately address this question. We could attempt to answer this question by isolating CD11b+ bone marrow derived monocytes (BMDM) from donor mice that express a GFP marker, treating them with pioglitazone, and injecting them into a recipient mouse by an intracardiac puncture or subcutaneous vascular port (Lebson et al., 2010). Animals would have to be sacrificed and brain sections would be stained for Iba1, to label all microglia and 6E10, to label Aβ. The number of Iba+/GFP+/6E10+ (infiltrating microglia/monocytes) cells would have to be counted and compared to Iba+/-GFP-/6E10+ (resident microglia) cells. If PPARγ activation primes infiltrating monocytes, which then immigrate into the brain differentiate into M2 microglia and efficiently phagocytose Aβ,
then all microglial cells that have internalized 6E10 deposits will be GFP positive. We expect that Iba1+/GFP− cells will be unable to take up significant quantities of Aβ since they have not been exposed to PPARγ agonist. We could then repeat this same experiment, but additionally treat the animal for 9 days with Pioglitazone treatment before injection of pioglitazone treated BMDMs. In this case, if Iba1+/GFP− cells are able to clear amyloid deposits, it would suggest that PPARγ can polarize resident microglia to a M2 state. FACS sorting can be utilized to sort out the populations of cells using markers indicated and accessing levels of M1 and M2 genes by qRT-PCR.

**PPARγ, proteases and amyloid beta**

*PPARγ mediated degradation of soluble Aβ by insulin-degrading enzyme and neprilysin*

Many proteases have been implicated in AD pathophysiology, a few of the most studied include: insulin degrading enzyme (IDE), neprilysin (NEP), endothelin-converting enzyme (ECE), angiotensin-converting enzyme (ACE) and plasmin (Wang et al., 2006). In the brain, Aβ peptides are principally degraded by IDE and NEP (Kurochkin and Goto, 1994, Iwata et al., 2001). Inhibition of these genes, either genetically or though inhibitors, results in elevated brain Aβ levels and increased plaque deposition (Iwata et al., 2001, Farris et al., 2003, Dolev and Michaelson, 2004, Farris et al., 2007). Similarly, overexpression of either NEP or IDE in an AD transgenic mouse model ameliorates plaque deposition and cognitive deficits (Leissring et al., 2003, Hemming et al., 2007, Meilandt et al., 2009).
We have previously documented that LXR activation promotes the proteolytic degradation of soluble Aβ extracellularly, through the actions of IDE, and intracellularly, by neprilysin (Jiang et al., 2008). Similarly, PPARγ-mediated degradation of soluble Aβ occurs dominantly through neprilysin, as inhibitor of neprilysin (Phosphoramidon: PRD) inhibits intracellular degradation of Aβ by both microglia and astrocytes (Figure 2). PPARγ activation has been shown to transcriptionally induce the expression of IDE in neurons, through a functional PPAR response element in the IDE gene promoter of neurons (Du et al., 2009b). Conversely, PPARγ antagonist treatment of an AD mouse model results in decreased levels of brain IDE (Du et al., 2009a). We did not observe any changes in transcript levels of nep or ide in cortical homogenates from pioglitazone treated APP/PS1 animals (Figure 3).

**PPARγ and the plasminogen system**

Another protease that has been associated with AD and PPARγ activation is plasmin. Although the plasmin system has been mainly characterized in regards to fibrinolysis and cell migration, a substantial body of literature has shown its role in Aβ degradation. In this proteolytic cascade, plasminogen is converted to the active protease plasmin by tissue plasminogen activator (tPA) (Van Nostrand and Porter, 1999, Periz and Fortini, 2000). Plasmin has been previously shown to cleave monomeric, oligomeric and fibrillar forms of Aβ (Tucker et al., 2000). All components of the plasmin system are known to be expressed in the CNS. Both tPA and PAI-1 are secreted by astrocytes as well as microglia while plasminogen is predominantly expressed by neurons (Tucker et al., 2000, Hino et al., 2001). The activity of plasmin and its activators are regulated via the activity the tPA inhibitor, plasminogen activator inhibitor (PAI-1). PAI-1 acts
physiologically as the primary inhibitor of the plasminogen activators (Gils and Declerck, 2004a, b). All components of this cascade have been shown to be codeposited in the AD brain with Aβ plaques. Interestingly, PAI-1 expression has been shown to increase in the presence of inflammatory stimuli such as TNF-α, IL-1β and TNF-β, all of which contribute to the pathology of AD (McGeer and McGeer, 1995, Hoo et al., 2007). Post-mortem and animal studies have shown that in an AD brain there is an elevation in brain levels of PAI-1 and a concurrent decrease in brain levels of tPA (Hino et al., 2001, Melchor et al., 2003). Both tPA and plasminogen have been shown to reduce Aβ-mediated toxicity. Furthermore, stimulation of primary cortical neurons with aggregated forms of Aβ results in an upregulation of tPA mRNA levels (Kingston et al., 1995, Lee et al., 2007).

Most importantly, small molecule inhibition of PAI-1 resulted in clearance of Aβ and improvement of behavior and cognitive impairment in two mouse models of AD (Tg2576 and PSAPP). More strikingly, these improvements were seen in both pre and post plaque-bearing animals (Jacobsen et al., 2008). These data strongly suggest that activation of plasmin cascade could reverse AD related plaque pathology and behavior. A more recent study has demonstrated that genetic loss of PAI-1 in an AD mouse model attenuates amyloid pathology (Liu et al., 2009). Of particular relevance to the present work is the observation that PAI-1 expression is negatively regulated by PPARγ activation (Hao et al., 2008).

In mixed glial cultures, LPS stimulation increases secreted levels of PAI-1 protein into the media. Treatment of these cells with pioglitazone dose dependently reduces levels of secreted PAI-1 from these cells suggesting that PPARγ activation may activate
the plasmin pathway though inhibition of PAI-1 protein levels (Figure 5). This pathway was not explored any further due to the limited availability of reagents that detect the protein and well as activity of these enzymes in mice. However, is likely that PPARγ activation in the APP/PS1 mouse model may exert some of its effects through the plasmin system. It would be interesting to see if levels of PAI-1 protein or mRNA are decreased in pioglitazone treated APP/PS1 animals and if this reduction is associated with increased tPA and plasmin enzymatic activity. This would be particularly exciting because plasmin has been shown to degrade all forms of Aβ.

**PPARγ and ApoE isoforms**

One of the significant findings in this research endeavor was the discovery of the ability of PPARγ to activate the LXR pathway in the brain, and induce ApoE expression, facilitating amyloid clearance. These studies are fairly easy to conduct in a murine model of AD, which only expresses one isoform of ApoE. Humans however, possess three ApoE isoforms which differ only by two amino acids, ApoE2, ApoE3 and ApoE4. ApoE2 is considered protective for AD while ApoE4 confers the highest susceptibility for AD (Kim et al., 2009). Studies with human ApoE knockin mice have shown a genotype dependent difference in amyloid deposition. Mice expressing ApoE4 display the highest levels of amyloid deposition while those expressing ApoE2 have significantly fewer deposits (Fagan et al., 2002). Additionally, ApoE isoforms affected the degradation of soluble Aβ in microglia in a similar fashion (Jiang et al., 2008). The ability of ApoE to become lipidaded is also dependent on ApoE isoform. ApoE2 and E3
containing particles have the ability to be loaded with large amounts of cholesterol and phospholipids forming larger HDL particles, while ApoE4 containing HDL particles are much smaller in size (Jiang et al., 2008) and are less efficient in promoting Aβ degradation.

For pioglitazone to exert its actions in humans it has to be able to promote ApoE mediated degradation of Aβ regardless of ApoE isoform. It will therefore be crucial to determine the effects of PPARγ activation on all three ApoE isoforms. Of most concern is the ApoE4 allele. Riddell et al, have demonstrated that ApoE4 containing particles are more rapidly degraded in comparison to ApoE2 and ApoE3 particles (Riddell et al., 2008). Since activation of PPARγ induces the expression of ABCA1 it would be interesting to see if enhanced lipidation of ApoE4 would increase particle size and extend the half-life of the protein.

PPARγ activation in astrocytes

One of the most important findings in this study is that pioglitazone treatment enhanced uptake and degradation of Aβ peptides by astrocytes. Astrocytes play a fundamental role in brain homeostasis, they provide metabolic support for neurons, form the blood-brain barrier, and possess a wide number of receptors that allows them to respond to variety of neurotransmitters, neuropeptides, growth factors and cytokines (Liberto et al., 2004, Fuller et al., 2009). Traditionally, microglia have been thought to be the primary immune effectors of the central nervous system thus little attention has been paid to astrocytes in AD.
We provide *in vitro* data demonstrating that astrocytes are capable of taking up soluble species of Aβ and degrading them in an ApoE dependent mechanism, similar to microglia. Additionally, we display that astrocytes are more sensitive to drug treatment and can take up and degrade Aβ as efficiently as microglial cells. This is important because microglia only comprise 5-10% of the total cells in the brain. Astrocytes are far more abundant and occupy more surface area than microglia, thus a small contribution on their part to lead to a large impact in amyloid clearance over the whole brain.

The most exciting finding, however, was that PPARγ agonist treatment of 12 month old APP/PS1 mice induced the astrocytic uptake of Aβ *in vivo* as visualized by confocal microscopy. While uptake of Aβ by astrocytes has been documented, we are the first to show a drug dependent effect on astrocytic uptake of Aβ (DeWitt et al., 1998, Matsunaga et al., 2003, Wyss-Coray et al., 2003, Koistinaho et al., 2004). Based on our data we postulate that astrocytes may be able to take up and clear Aβ through two independent pathways, similar to microglia. Through one mechanism they utilize ApoE dependent degradation of soluble Aβ species and a second would allow for uptake of larger particle, possibly through a phagocytic mechanism. Most importantly, our data suggests that the role of astrocytes in AD related pathology and drug treatment must be re-evaluated.

**PPARγ activation through RXRs**

RXRs play a central role in nuclear receptor biology due to their heterodimerization with many other type II nuclear receptor family members, including LXRs
and PPARs (Mangelsdorf and Evans, 1995, Szanto et al., 2004). Due to its central role in nuclear receptor signaling and its interactions with both PPAR and LXR, it is hypothesized that RXR activation would simultaneously activate both LXR and PPAR signaling pathways and initiate ApoE mediated clearance pathways. Studies in our laboratory have shown that treatment of APP/PS1 animals with the RXR agonist bexarotene for only 3 days results in a dramatic induction of ApoE and ABCA1 protein levels and this associated with a rapid reversal of AD-associated pathological hallmarks and cognitive deficits.

In addition, it would be very interesting to see if RXR activation through agonists such as bexarotene and honokiol, will result in microglial polarization effects seen by using the PPARγ agonist, pioglitazone. This would be very exciting because the RXR agonist, bexarotene, is FDA-approved, has a positive side-effect profile, and can efficiently permeate the BBB (data not shown).

**Other functions of PPARγ**

PPARγ activation, by pioglitazone has also been shown to rescue cerebrovascular function in an aged AD mouse model. A study conducted on 14 month old APP V717I animals treated with 20mg/kg/day of pioglitazone for 6-8 weeks showed that PPARγ activation rescued neurometabolic coupling and cholinergic denervation. These authors observed no effects of pioglitazone on plaque load in these animals, but this could be attributed to the low drug dose utilized in this study (Nicolakakis et al., 2008).
PPARγ activation has also been postulated to play a role in restoring dendritic spine density. Cultured primary cortical rat neurons show a dose-dependent increase in dendritic spine density when treated with rosiglitazone. These results were abolished with a PPARγ specific antagonist. Rosiglitazone was also able to restore the loss in spine density cause by the ApoE4 protein (Brodbeck et al., 2008).

While we have demonstrated two active mechanisms through which PPARγ facilitates amyloid clearance. Other studies have documented other mechanisms through which PPARγ may also ameliorate AD pathology and it is important to keep these mechanisms in mind to truly understand the full range of PPAR actions in the brain.

Clinical Trials of PPAR agonists

Many studies have now documented the beneficial effects of PPARγ activation is AD (Yan et al., 2003, Heneka et al., 2005, Escribano et al., 2010, Toledo and Inestrosa, 2010, Rodriguez-Rivera et al., 2011). Owing to the promising effects on AD mouse models, a small clinical trial was conducted to determine the efficacy of pioglitazone in patients exhibiting signs of mild to moderate AD. In this study pioglitazone treatment was shown to improve memory and cognition in these patients (Hanyu et al., 2009, Sato et al., 2009). Recently, the effect of rosiglitazone in treating AD was examined in larger clinical trials. A phase II clinical trial, where patients treated with rosiglitazone for 6 months, showed improvements in attention and memory retention, but only in patients that did not have an ApoE4 allele. However, much larger phase III trials failed to show
any efficacy in mild to moderate AD patients (Risner et al., 2006, Gold et al., 2010). It is important to note, that rosiglitazone is known to have very poor BBB permeability and was administered at approximately ten percent the dosage known to be efficacious in rodent models of AD. Additionally, rosiglitazone is a substrate for p-glycoprotein, promoting its efflux from the brain into peripheral circulation (Hemauer et al., 2010). Thus, due to the design of these trials it is unclear whether existing PPARγ agonists will be beneficial in treatment of AD. It is our view that PPARγ activation remains a promising avenue for the treatment for AD.

Conclusions

In conclusion, our results demonstrate the ability of PPARγ to activate two mechanisms through which rapid amyloid clearance is achieved in a mouse model of AD. Through one mechanism, PPARγ activation results in the induction of LXR target genes, ApoE and ABCA1, and mediates the degradation of soluble Aβ by increasing the abundance of lipidated ApoE particles. Through a second mechanism PPARγ activation serves to polarize the microglial population, reducing the production of pro-inflammatory cytokine, enhancing their phagocytic abilities resulting in a rapid reversal of AD pathology. Additionally, PPARγ activation also facilitates amyloid degradation by astrocytes. Altogether, these data suggests that targeting activation of PPARγ serves as a promising therapeutic avenue for the treatment of Alzheimer’s disease.
Figure 1: PPARγ activation influences the inflammatory milieu of the brain.

Quantitative PCR results from the brains of vehicle treated wildtype (WT) and APP/PS1 animals (Veh) and pioglitazone treated APP/PS1 animals (Pio) for inflammatory cytokines at 6 months (A) or 12 months (B) of age. (mean ± SEM, Student’s T test, *p<0.05, **p<0.01, ***p<0.001, n≥6 animals per group).
Figure 2: PPARγ activation mediates intracellular degradation of Aβ via Neprilysin. Primary microglia (A) or astrocytes (B) were isolated from wildtype ice (p0-P3) and pretreated with neprilysin inhibitor phosphoramidon (10μM) prior to the the addition of either DMSO (ctrl) or Pioglitazone for 24 hrs. The cells were then incubated for 24 hrs with soluble Aβ42 in the presence or absence of drug and NEP inhibors. Intracellular Aβ levels were quantified using ELISA for Aβ42 and normalized to total protein. (mean ± SEM, Student’s T test, *p<0.05, **p<0.01, ***p<0.001, n=4).
Figure 3: PPARγ activation does not affect mRNA expression of IDE or Neprilysin.
Quantitative PCR results from the brains of vehicle treated wildtype (WT) and APP/PS1 animals (Veh) and pioglitazone treated APP/PS1 animals (Pio) for Aβ proteases at 6 months (A) or 12 months (B) of age. (mean ± SEM, Student’s T test, *p<0.05, **p<0.01, ***p<0.001, n≤13 animals per group).
Chapter 5

Literature Cited


