Interleukin-1 (IL-1) as a potential contributor to the pathophysiology of Alzheimer's Disease

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Interleukin-1 (IL-1) as a potential contributor to the pathophysiology of Alzheimer's Disease

by

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Abstract

Interleukin-1 (IL-1) as a potential contributor to the pathophysiology of Alzheimer's Disease

By

Sara Nicole Resch

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Elevations of the pro-inflammatory cytokine interleukin-1 (IL-1) have been reported as part of the inflammatory response to injury and neurodegenerative disorders. The observation of increased IL-1 levels in the brains of patients with Alzheimer’s disease (AD) triggered over two decades of research implicating IL-1 in the disease pathogenesis. IL-1 has been suggested to modulate a variety of actions related to AD pathology, including the processing of the Aβ precursor protein and deposition of amyloid plaques. IL-1 also activates glial cells and induces recruitment of leukocytes and peripheral macrophages to the CNS across the blood-brain barrier (BBB). When exposed to IL-1, microglia and macrophages take on a classical activation phenotype resulting in the release of more inflammatory cytokines and reactive oxygen species that can cause cell degeneration. In addition, IL-1 expression has been shown to impair hippocampally-mediated memory processes. In fact, imaging studies with AD patients have demonstrated closer correlations between cognitive function and activated microglia than between cognitive function and amyloid plaque burden. Epidemiological studies reveal a reduced risk of developing AD for chronic users of anti-inflammatory drugs, but clinical trials have not shown a consistent benefit of NSAID treatment. In this study, we attempted a more targeted approach to anti-inflammation treatment through chronic, systemic administration of the mIL-1 Trap, which specifically inhibits IL-1 signaling, to transgenic swAPP/PS1 mice. After 5 months of treatment, animals that were treated with the mIL-1 Trap performed significantly
better on water maze acquisition, a measure of learning, than transgenic animals treated with mFc (placebo). However, the hippocampal plaque burden (number of plaques, average plaque size, and proportion of hippocampus covered with plaques) was unchanged by the treatment. We also found an effect of the mIL-1 Trap treatment on microglial morphology in the hippocampus. Animals treated with mIL-1 Trap had larger somas for microglia adjacent to amyloid plaques but smaller somas for microglia that were distant from plaques. Finally, correlations between the plaque measurements, cognitive performances, and microglial measurements revealed differences between the two treatment groups. These findings may shed more light on the relationship between interleukin-1, neuroinflammation, and plaque pathology in Alzheimer’s disease.
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Chapter 1

INTRODUCTION

Interleukin-1

Interleukin-1 (IL-1) is a protein that is part of a larger family of inflammatory and regulatory cytokines with especially important roles in regulation of the immune system. IL-1 is found in two distinct isoforms, IL-1α and IL-1β, although IL-1β is considered the primary active isoform. They are both synthesized as large precursor proteins by cells in the central nervous system (CNS) as well as by peripheral cells, such as lymphocytes and monocytes (Allan, Tyrrell, & Rothwell, 2005). Mature IL-1 is formed by cleavage of its proprotein, Prol-IL-1. Pro-IL-1α is active on its own, but Pro-IL-1β is biologically inactive and must be cleaved by Caspase-1 or IL-1β converting enzyme (ICE) in order to produce the active IL-1β protein (Thornberry & Molineaux, 1995). Active IL-1β (hereafter simply referred to as IL-1) is a secreted protein that can be released to induce or augment inflammatory responses throughout the body.

IL-1 exerts its biological actions through the cell surface receptor IL-1R1, which is expressed in many cells throughout the body, including in the brain where the highest levels are observed in the pyramidal cell layer of the hippocampus, dentate gyrus, cerebellum, pituitary gland, and hypothalamus (Farrar, Kilian, Ruff, Hill, & Pert, 1987; Cunningham et al., 1992). IL-1 agonists bind to the receptor and cause downstream effects due to association with an accessory protein. In addition to the IL-1 family agonists, many cells produce IL-1ra, an endogenous antagonist to IL-1R1 that binds to the receptor but does not trigger the receptor association with its accessory protein, therefore competitively blocking IL-1 signaling through IL-1R1 (for reviews see Allan et al., 2005; Patel, Boutin & Allan, 2003).
IL-1 and the Inflammatory Response

IL-1 is one of the most widely studied inflammatory mediators. In addition to its role in more traditional immune diseases, it has been implicated in a wide range of acute and chronic neurodegenerative conditions. IL-1β is an important factor in neuroinflammation, including in the CNS's response to pathogens, injury and cell death. The innate neuroinflammatory response is characterized by activation of resident glial cells (microglia and astrocytes), infiltration of peripheral immune cells (leukocytes), production of the enzyme cyclooxygenase-2 (COX-2), and the expression of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α (Basu et al., 2002). IL-1β is primarily produced by microglia, the resident macrophages of the CNS (Van Dam, Bauer, Tilders, & Berkenbosch, 1995). Microglia respond to pathogen-associated molecular patterns (PAMPs) through pathogen recognition receptors, such as toll-like receptors (TLR). In response to a PAMP, the TLR becomes activated and begins gene transcription of caspase-1, resulting in a surge of IL-1 in the brain (Eriksson et al., 1999).

IL-1 and other cytokines released in the early innate stage of the immune response activate the later adaptive immune response. The adaptive response involves activation and recruitment of peripheral leukocytes, including lymphocytes, to the site of the injury (Carpentier & Palmer, 2009). The pathogens and affected cells are then removed through antigen presentation and recognition, as well as production of antibodies and effector T and B cells (Varnum & Ikezu, 2012; Carpentier & Palmer, 2009). IL-1 also activates the hypothalamic-pituitary-adrenal axis (HPA axis) to release glucocorticoids, which may act as anti-inflammatories and suppress the release of pro-inflammatory proteins (Silverman, Pearce, Biron, & Miller, 2005). As the initiating PAMPs are cleared, the immune responses are resolved through a decrease in pro-inflammatory cytokines and an increase in glucocorticoids and anti-
inflammatory cytokines like IL-4, IL-10 and TGF-β. This negative feedback control prevents an overactive inflammatory response and restores homeostasis (Silverman et al., 2005).

**IL-1 in Acute CNS Injury**

In the normal brain, expression of IL-1 is low, but acute CNS injury causes a sharp increase in IL-1 production. Elevated levels of the cytokine are found in the cerebrospinal fluid of patients with a range of CNS injuries, including infections, stroke, and traumatic injuries (Patel et al., 2003; Allan et al., 2005). In rodents, the experimental induction of both ischemia and excitotoxicity rapidly stimulates an increase in expression of IL-1β protein and mRNA (Liu et al., 1993; Pearson, Rothwell, & Toulmound, 1999).

There is evidence to suggest that IL-1β plays a causal role in the cell death observed in CNS injury. The time course of IL-1 expression provides indirect evidence. For example, stroke, head injury and ischemia are acute events, but cell degeneration can occur hours to days after the initial injury, corresponding to the release of IL-1β and other pro-inflammatory cytokines (Allan & Rothwell, 2001). Though IL-1β by itself does not cause cell death, it contributes to neurodegeneration. When IL-1β is injected along with experimental induction of ischemia, it markedly increases cell damage (Loddick & Rothwell, 1996; Stroemer & Rothwell, 1998). Acute increases of IL-1β can exacerbate excitotoxicity induced by NMDA receptor agonists, partly by stimulating glial production of nitric oxide (Morimoto, Murasugi, & Oda, 2002). Further evidence comes from the inhibition of IL-1 during CNS injury, which has protective effects. Blocking IL-1 through the injection of IL-1ra (Loddick & Rothwell, 1996; Stroemer & Rothwell, 1997) or an ICE inhibitor (Loddick, MacKenzie, & Rothwell, 1996) reduces cell death associated with ischemia and traumatic brain injury in rodents.
IL-1 in Infection

IL-1 and other pro-inflammatory cytokines play a key role in the immune response to infection. IL-1 is involved in producing symptoms of infection, such as fever and hyperalgesia and behavioral changes including lethargy, anorexia, and decreased activity (Kelley et al., 2003). These symptoms, called “sickness behavior” are part of a motivational state in which behavior is organized to optimize fighting off the infection. Fever, an increase in the regular set point of body temperature, causes the organism to seek warmer temperatures and reduce heat loss, inhibiting the growth of pathogens. Since the metabolic cost of fever is high, lethargy forces the individual to rest and conserve energy for fighting the pathogen (Dantzer, 2001).

Infection is often experimentally induced through administration of Lipopolysaccharide (LPS), a component of Gram-negative bacteria, which increases expression of IL-1α and IL-1β in the brain (van Dam et al., 1998). Injections of LPS or cytokines into either the periphery or the lateral ventricle of the brain results in decreased locomotor activity, reduced food intake, and reduced grooming (Kent et al., 1992). Intracerebroventricular (ICV) pretreatment with IL-1 receptor antagonist blocks some behavioral symptoms of either ICV or peripheral injection with IL-1β (Bluthe, Dantzer, & Kelley, 1992). Aged mice show prolonged and exaggerated sickness behavior in response to LPS, corresponding to greater increases of pro-inflammatory cytokines and microglial activation markers (Huang, Henry, Dantzer, Johnson, & Godbout, 2008).

It is important to note that CNS production of IL-1 occurs even in response to infections in the periphery. Intraperitoneal injections of LPS induce the expression of the pro-inflammatory cytokines IL-1 and TNF-α followed later by IL-6 and IL-1ra in the brain (Laye, Parnet, Goujon, & Dantzer, 1994). The finding that ICV IL-1ra inhibits sickness behavior induced by peripheral IL-1 suggests that the symptoms of peripheral infection are probably centrally mediated (Bluthe
et al., 1992). Peripheral LPS or IL-1β stimulates the vagus nerve, leading to activation in the nerve’s primary and secondary projection areas and activation of the HPA axis (Dantzer, 2001). In addition to this fast neural pathway, IL-1β signals the brain via a slower humoral pathway. IL-1β production in the brain first occurs in circumventricular organs, such as the area postrema, and then spreads into the rest of the brain. The IL-1β then activates parenchymal structures, recruits microglia and induces expression of iNOS (Konsman, Kelley, & Dantzer, 1999).

Alzheimer’s Disease

IL-1-mediated neuroinflammation has been implicated in the pathogenesis of several neurodegenerative diseases, including Alzheimer’s disease (AD). AD is the most common form of dementia and it presents with progressive decline of cognitive functioning in elderly populations. Symptoms of AD have an insidious onset over the course of months or years and progressively worsen. The most commonly affected cognitive domain is memory, with a progressive inability to learn and recall new information, but other cognitive functions can also be affected, such as language and executive functioning (McKhann et al., 2011). Neuropsychiatric symptoms are prevalent in patients suffering from the disease. Of these symptoms, depression and anxiety are particularly common (Di Iulio et al., 2010), along with agitation (Porter et al., 2003; Mega, Cummings, Fiorello, & Gornbein, 1996).

The characteristic pathology of AD is the intraneuronal accumulation of hyperphosphorylated tau and extracellular deposits of Aβ in the form of amyloid plaques (Alzheimer et al., 1995; Dickson et al., 1988). AD also is associated with a loss of neurons and synapses, leading to atrophy especially in the frontal and temporal cortices (Davies, Mann, Sumpter, & Yates, 1987). Neurotransmitter levels and function are also altered in AD, with
cholinergic function being one of the earliest and most severely impacted due to decreased choline acetyltransferase activity and the loss of forebrain cholinergic neurons (Wenk, 2003).

**Amyloid Cascade Hypothesis**

A majority of research on AD to date has focused on the role of amyloid β (Aβ) in cognitive and neuronal dysfunction. Amyloid β peptide is formed by cleavage of amyloid precursor protein (APP) first by β-secretases and then γ-secretases (Citron et al., 1996; Seubert et al., 1993). Several isoforms of different lengths can be created, but the most common isoforms are Aβ40 and Aβ42. While the soluble Aβ40 fragment is found in the cerebrospinal fluid of both AD patients and normal controls (Seubert et al., 1992, Shoji et al., 1992), the Aβ42 fragment is more concentrated within neuritic plaques, and has therefore been more closely associated with the disease (Roher et al 1993). (See Figure 1)

The discovery of Aβ within senile plaques led to the “amyloid cascade hypothesis,” (ACH) which designates Aβ deposition as the initiating event in AD pathogenesis. According to the amyloid cascade hypothesis, abnormal processing of APP results in overproduction of Aβ42 fragments which cannot be cleared effectively and accumulate in the brain (Hardy & Higgins, 1992). This deposition of Aβ is thought to be the initial event that causes the variety of changes seen in the AD brain, including neurofibrillary tangles, neuronal death and cognitive decline (Hardy & Higgins 1992; Reitz, 2012). The ACH is also supported by the link between early-onset familial dementia and mutations in genes responsible for APP (Citron et al., 1992), as well as genes for presenilin1 (PS1) and PS2 which are components of the γ-secretase complex (Sherrington et al. 1995). (Figure 1)
Figure 1. APP processing. Amyloid β is produced by the cleavage of APP, first by the β-secretase followed by the γ-secretase. Gamma-secretase can cleave at alternate sites to produce either Aβ_{40} or Aβ_{42}. Multiple known mutations that affect APP processing and Aβ production can occur at various sites along APP and are linked with familial AD. For example, the Swedish mutation is a well-known familial mutation that occurs at the beta-secretase cleavage site. Several other mutations occur at the gamma secretase cleavage site. Mutations of gamma-secretase itself, such as PS1Δe9, in which exon 9 of the presenilin1 component is deleted, result in greater concentrations of harmful Aβ_{42} relative to Aβ_{40}. Many of these mutations have been used to engineer transgenic mouse models of AD. (Figure adapted from Hall and Roberson, 2012).

Despite the apparent involvement of Aβ in AD, several findings challenge the amyloid hypothesis. First, clinico-pathologic studies have reported finding a high Aβ burden in the brains of healthy individuals who do not qualify for a dementia diagnosis (Aizenstein et al., 2008; Lue, Brachova, Civin, & Rogers, 1996; Lue et al., 1999). Although they likely represent a preclinical stage of the disease, these high pathology, non-demented individuals show little evidence of cortical synapse loss characteristic of neurodegenerative disorders (Lue et al., 1996). A second challenge to the amyloid hypothesis comes from positron emission tomography (PET) studies
using Pittsburgh Compound B (PIB), a marker for Aβ. On one hand, PIB uptake is significantly increased in several cortical areas in patients diagnosed with AD relative to healthy controls and some studies have identified an association between PIB uptake and clinical dementia severity (Grimmer et al., 2009). However, studies looking at the relationship between Aβ load and cognitive function (using either the mini mental status examination or a battery of neuropsychological memory tests) have found either a weak correlation (Villemange et al., 2011) or no correlation (Edison et al., 2008; Yokokura et al., 2011) suggesting that symptom progression is due to downstream factors. Finally, treatments that target and remove Aβ deposits, including active anti-Aβ vaccines (Holmes et al., 2008), passive anti-Aβ monoclonal antibodies (Salloway et al., 2009, Salloway et al., 2014; Doody et al., 2014), and gamma secretase inhibitors (Doody et al., 2013) have failed to stop progressive neurodegeneration and cognitive decline in AD, even when successfully clearing amyloid plaques from the brain.

**Alzheimer’s Disease and Inflammation**

An alternative to the amyloid cascade hypothesis is that the neuron degeneration in AD is due not to the Aβ plaques themselves, but to a dysregulated inflammatory response, perhaps triggered by or aggravated by the amyloid deposits (McGeer, Rogers, & McGeer, 1994). There is mounting evidence that implicates inflammation as a causal factor in disease progression. This hypothesis is based on the finding of robust neuroinflammation in AD brains, characterized by microglial and astrocytic activation and the upregulation of inflammatory markers such as cytokines and chemokines (Shaftel, Griffin, & O’Banion, 2008). Inflammatory markers are co-localized to brain areas that are most affected by AD pathology such as the limbic cortex and frontal lobes and tend to be expressed near amyloid plaques and neurofibrillary tangles.
(Akiyama et al., 2000). Inflammatory activation occurs very early in the disease progression, often before other symptoms are observed. In transgenic AD mice, microglial and astrocytic activation along with pro-inflammatory cytokine expression is observed before the formation of amyloid plaques (Heneka et al., 2005).

Imaging studies have demonstrated a relationship between inflammation, as measured by microglial activation, and AD symptom severity. A PET and MRI study of patients with early AD showed an age-dependent increase in microglial activation relative to healthy controls, correlating with areas of atrophy (Cagnin et al., 2001). Two PET studies using PK 11195, a microglial marker, found a negative correlation between performance on neuropsychological testing and microglial activation in the cortices of AD patients (Edison et al., 2008; Yokokura, 2011). These same studies failed to find a significant correlation between cognitive functioning and Aβ load using PIB.

Components of the Inflammatory Profile in AD

Interleukin-1. IL-1 is an integral component of the inflammatory profile in AD. In 1989, Griffin and colleagues first reported elevated levels of IL-1 in the brain tissue of patients with AD, including in IL-1 reactive microglia. Since then, a subset of AD research has focused on the relationship between interleukin-1 mediated neuroinflammation and disease pathogenesis. Elevations of IL-1 have also been demonstrated in rodent models of the disease (Benzing et al., 1999).

IL-1 has been implicated as a key factor in promoting Aβ plaque pathology. Overexpression of this cytokine is observed very early in the course of the disease, before neuritic plaques are fully formed. The number of IL-1 reactive microglia correlates with the
number of dystrophic neurites found in Aβ plaques (Griffin, Sheng, Roberts, & Mrak, 1995). IL-1 directly contributes to amyloid deposition by promoting the synthesis of APP (Goldgaber et al., 1989), and transforming diffuse β-amyloid aggregates into mature β-amyloid plaques (Griffin et al., 1995). In APP<sub>swe</sub> mice, LPS-induced neuroinflammation increases the expression and processing of APP and the accumulation of intracellular Aβ peptides (Sheng et al., 2003). In one study, transgenic mice that expressed serum amyloid A, an acute phase protein, in the brain did not show amyloid deposition unless injected with LPS, which increased levels of IL-1, IL-6 and TNF-α (Guo, Yu, Grass, de Beer, & Kindy, 2002). The amyloid deposition in the brain was significantly reduced when the LPS–treated animals were concurrently treated with a nonsteroidal anti-inflammatory agent.

In AD, the chronic upregulation of IL-1β points to a dysregulated immune system in which the innate pro-inflammatory response is unchecked. While IL-1 may promote Aβ synthesis and deposition, Aβ itself can activate glial cells, inducing further production of IL-1β. The result is more Aβ synthesis and deposition and increased neuronal injury, which causes even more IL-1β to be released. This positive feedback amplifies the IL-1 expression and causes a self-propagating “cytokine cycle” (Griffin et al., 1998).

IL-1 is also involved in promoting cholinergic dysfunction in AD. Acetylcholinesterase (AChE), a postsynaptic enzyme that hydrolyzes acetylcholine at the synapse, is overactivated in AD brains, especially by neurons associated with plaques. Implanting rat cortex with IL-1β pellets induced an increase in AChE mRNA levels (Li et al., 2000). In vitro, microglial cultures secreted IL-1 in response to fragments of APP, stimulating primary neuron cultures to upregulate AChE activity (Li et al., 2000).
**Microglia.** In AD, the inflammatory profile is characterized by increased numbers of activated microglia, especially surrounding amyloid plaques. In normal brains, microglia are distributed throughout the gray and white matter, but in the brains of humans with AD and also in transgenic mouse models of AD, the microglia cluster around deposits of Aβ (El Khoury & Luster, 2008). These plaque-associated microglia express significantly more IL-1 compared to microglia in normal brains and non-plaque associated microglia in AD brains (Griffin et al., 1989).

Microglia can exist in several levels of activation. In their quiescent state, they provide surveillance for surrounding neurons (Lue, Kuo, Beach, & Walker, 2010; Town, Nikolic, & Tan, 2005). In the presence of injurious stimuli, microglia react quickly, releasing many substances such as cytokines, chemokines, reactive oxygen species and adaptive immune-function molecules. Microglia can also exist in a primed or sensitized state. Normally, microglia will revert to the quiescent state after the body returns to homeostasis, but they can remain in a chronic primed state when low levels of stimulation are present in the microenvironment (Lue et al., 2010). In the primed state, they express several activation markers, such as major histocompatibility complex II (MHC II), but low levels of pro-inflammatory cytokines. However, in the presence of an immune stimulus, the resulting cytokine release is amplified and prolonged (Wynne, Henry, & Godbout, 2009).

Microglial priming occurs in normal aging as part of a shift in the CNS microenvironment from an anti-inflammatory to pro-inflammatory profile. In normally aging brains, there is an upregulation of MHC II along with a decrease in mRNA expression of the proteins IL-10 and CD200, which down-regulate glial activation (Frank et al., 2006). Primed microglia in the aged brain are sensitized to produce exaggerated responses to additional stimuli.
For example, peripheral infection produces a dramatic long-term increase in IL-1β in the hippocampus of aged rats, but not in young rats, even though basal levels of IL-1β are not consistently affected by age alone (Barrientos et al., 2009). In AD, age and amyloid deposition could potentially combine to produce microglial activation and release of cytokines. In aged AD transgenic animals relative to young animals and controls, LPS produces an exaggerated increase in IL-1β levels in the hippocampus but not cerebellum where there are few amyloid plaques (Sly et al., 2001). This finding suggests that both age and Aβ plaque accumulation prime microglia leading to the heightened inflammatory state observed in AD.

It has recently been acknowledged that microglia (and macrophages in general) can take on different activated phenotypes in response to different CNS insults. The M1 state, or classical pro-inflammatory activation state, is induced by Th1 cytokines such as IFN-γ, TNF-α and IL-1β (Varnum & Ikezu, 2012). Classically activated microglia release high levels of pro-inflammatory cytokines as well as reactive oxygen and nitrogen species, which kill pathogens but also lead to cell damage (Colton, 2009). In contrast, M2 activation, also known as alternative microglial activation, is induced by Th2 cytokines such as IL-4, IL-10 and IL-13 (Gordon, 2003). M2 microglia express arginase 1, which inhibits NOS production; mannose receptor 1, which activates anti-inflammatory signaling; and the genes FIZZ1 and YM1, which code for proteins that help reconstruct the extracellular matrix (Colton et al., 2006). In the AD brain, a shift from an M2 to M1 microglial activation is observed as the disease progresses. As APP/PS1 transgenic AD mice age, more microglia in the hippocampus take on the classically activated phenotype (Jimenez et al., 2008).

**Astrocytes.** Astrocytes also contribute to AD pathology. There is a positive correlation between PIB binding of amyloid in PET scans of AD patients and amount of glial fibrillary
protein-immunoreactive astrocytes upon autopsy (Kadir et al., 2011). In AD brains, astrocytes are found surrounding both diffuse and neuritic Aβ plaques (Alarcon, Fuenzalida, Santibanez, & von Bernhardi, 2005). Astrocytes in the entorhinal cortex accumulate neuronal material positive for Aβ42 with the amount of material correlating to the level of local AD pathology (Nagele, D’Andrea, Lee, Venkataraman, & Wang, 2003). Within the astrocytes, the amount of Aβ correlates with neuron-specific proteins such as choline acetyltransferase, suggesting that the astrocytic amyloid is acquired via phagocytosis of neuronal debris (Nagele et al., 2003). In vitro, astrocytes are able to degrade and clear amyloid peptides in solution, but they are unable to clear focal plaque-like deposits (Shaffer et al., 1995). The presence of activated astrocytes can even inhibit microglial phagocytosis of amyloid plaque cores, perhaps allowing the plaques to accumulate in AD (DeWitt, Perry, Cohen, Doller, & Silver, 1998; Shaffer et al., 1995).

Interleukin-1 can interact with astrocytes to promote AD pathology. IL-1α and IL-1β increase synthesis of APP by up to 6-fold in primary human astrocytes by enhancing APP mRNA translation (Rogers et al., 1999). IL-1 also causes astrocytes to overproduce s100β, a neurotrophic factor responsible for promoting neurite growth (Mrak & Griffin, 2001). In fact, increased s100β activity is seen in the temporal lobes of AD brains (Marshak, Pesce, Stanley, & Griffin, 1992). There is a positive correlation between the number of plaque-associated astrocytes expressing s100β and the amount of dystrophic neurite burden in the plaques, suggesting that s100β is contributing to the pathogenesis of neuritic plaques (Mrak, Sheng, & Griffin, 1996).

**Leukocyte recruitment.** Another component of the neuroinflammatory response is the recruitment of peripheral leukocytes, such as monocytes, to the CNS. In the normal adult brain, there are few leukocytes due to the presence of the blood brain barrier. However, under
pathological conditions such as in AD, monocytes and macrophages from the blood or bone marrow enter the CNS and differentiate into microglia-like cells (Prinz, Priller, Sisodia, & Ransohoff, 2011; Stalder et al., 2005). The recruitment of peripheral cells to the brain is mediated by cytokines. In mice, experimental elevations of IL-1β cause several types of leukocytes to migrate across the blood brain barrier (Shaftel et al., 2007a). These cells express microglial markers but may have important functional and morphological distinctions (Simard, Soulet, Gowing, Julien, & Rivest, 2006; Stalder et al., 2005).

Macrophages, like microglia, can be divided into classical (M1) and alternative (M2) activation subsets. M1 macrophages are thought to be actively recruited from peripheral circulation to the site of inflammation and to generate pro-inflammatory innate immune responses (Gate, Rezai-Zadeh, Jodry, Rentsendorj, & Town, 2010). M2 macrophages can be further broken down into M2a, M2b, and M2c subsets. M2a macrophages are phagocytic and anti-inflammatory effector cells (Colton et al., 2006). M2b macrophages participate in the clearance of reactive oxygen and nitrogen species (Gate et al., 2010). M2c macrophages are involved in tissue healing (Varnum and Ikezu, 2012). Some have suggested that it is these infiltrating peripheral macrophages, rather than resident microglia, that are driving the differential classical vs. alternative activation types (Ransohoff & Brown, 2012).

In the past, microglia and non-resident monocytes were referred to interchangeably, but recent research has begun to focus on the importance of distinguishing between the two. In a study using their IL-1β\textsuperscript{XAT} transgenic mouse model, which triggers overproduction of IL-1β when activated, Shaftel et al. (2007a) demonstrated that experimental increases of IL-1β result in an influx of leukocytes into the hippocampus, but no signs of neurodegeneration. In fact, studies show that peripherally recruited macrophages are better able to phagocytose and clear amyloid
plaques than their resident microglia counterparts (Simard et al., 2006). In transgenic AD mice, genetically blocking TGFβ signaling induced an influx of M2 activated macrophages to the brain, significantly increasing cerebral Aβ clearance (Town et al., 2008). These results suggest that recruitment of peripheral macrophages expressing an M2 phenotype could be beneficial in the treatment of AD pathology.

**Blood Brain Barrier Breakdown.** Related to the recruitment of peripheral cells to the CNS is the finding of increased permeability of the blood brain barrier (BBB) in Alzheimer’s disease and to a lesser extent, normal aging. The BBB is characterized by endothelial cells connected by tight junctions and is a highly selective barrier separating the CNS from peripheral circulation. Minogue et al. (2014) found an age-related increase in BBB permeability in the hippocampus of APP-PS1 transgenic mice using both magnetic resonance imaging and immunostaining for fibrinogen, which can accumulate in the brain across a damaged BBB. The transgenic mice also showed greater BBB disruption compared to wild-type mice as well as increased numbers of infiltrating immune cells, suggesting that vascular disruption and BBB breakdown contributes to the migration of peripheral leukocytes into the brain. Another study using rats showed that injecting them with Aβ1-42 resulted in increases in fibrinogen and microgliosis, whereas concurrently inhibiting microglia with anti-Mac-1 resulted in a relatively more intact BBB (Ryu and McLarnon, 2009). These findings suggest that glial cells interact with the vasculature in AD to maintain neuroinflammation.

Autopsy studies of human AD brains have also demonstrated evidence of BBB dysfunction. Fiala et al (2002) showed disruption in ZO-1, an endothelial tight junction protein, in association with peripheral macrophages at the site of weakened vessels. Ryu and McLarnon (2009) found greater fibrinogen leakage and immunoglobulin (IgG) reflective of a weakened
BBB in AD brains compared to normal controls. A recent study using an advanced dynamic contrast-enhanced MRI to look at regional BBB permeability found an age dependent breakdown beginning in the hippocampus that is present in normal aging, but more pronounced in MCI (Montagne et al., 2015). This finding suggests that increased BBB permeability is an early contributor to cognitive dysfunction in AD.

**Is Inflammation in AD Beneficial or Harmful?**

There has been some debate over whether IL-1 driven inflammation is protective or detrimental. As described above, IL-1β has been shown to contribute to cell death, but IL-1 expression in the brain can produce adaptive responses as well. In one study, exposing neuronal cultures to high levels of IL-1β was neurotoxic, but low levels of IL-1β actually protected against excitatory amino acid-induced cell death (Strijbos & Rothwell, 1995). Some have argued that IL-1 plays a beneficial role in AD. Although cytokines may promote amyloid formation and deposition, some studies show that that IL-1 reduces plaque pathology. In one study, Shaftel et al. (2007b) crossed their IL-1βXAT mouse model, which contains a transgene that triggers overproduction IL-1β in the hippocampus, with swAPP-PS1 transgenic mice, a mouse model of Alzheimer’s-like pathology. They found a significant reduction in plaque pathology 4 weeks after sustained IL-1β overexpression following transgene activation.

The proposed protective mechanism of IL-1 is through phagocytosis of amyloid plaques by microglia and macrophages, which could be viewed as an M2 response, although all microglia have phagocytic potential. The clustering of IL-1β -expressing, activated microglia around amyloid plaques suggests an attempt at phagocytic removal of the Aβ (Griffin et al., 1989). In vitro, microglia are effective in their phagocytic uptake of both amyloid aggregates
Microglia in the AD brain contain fragments of Aβ, supporting their clearance and processing role (D’Andrea, Cole & Ard, 2004). In early stages of the disease, microglial activation and recruitment of monocytes from the periphery seem to delay the progression of AD-like pathology (El Khoury & Luster, 2008; Simard et al., 2006). However, amyloid plaques continue to build up, despite the continued microglial activation.

Microglia and monocyte-derived macrophages can exist in several activated phenotypes that are determined by the local environment. The progression of most CNS diseases is from a primarily inflammatory M1 phenotype to a more phagocytic M2 phenotype. However, in chronic disease M1 activation is sustained. Microglia in the AD brain have reduced expression of Aβ-binding receptors and Aβ degrading enzymes, but still secrete pro-inflammatory cytokines (Hickman, Allison, & El Khoury, 2008). In this state, phagocytosis is impaired and Aβ clearance is reduced. In addition to their decreased Aβ-clearing effectiveness and IL-1β production, the activated microglia also continue to produce reactive oxygen and nitrogen species that are neurotoxic and cause neuronal degeneration (Weldon et al, 1998). The shift may be caused by the presence of amyloid, which interacts with toll-like receptors and stimulates microglial release of NO and TNFα (Lotz et al., 2005).

In other words, it may not be the strength of the immune activation, but the type of response that is important. In human microglia cultures, activating an M1 phenotype inhibits Aβ clearance, but adding the anti-inflammatory cytokines IL-4 or IL-10, which drive the M2 phenotype, promotes Aβ degradation (Yamamoto et al., 2008). Medeiros et al. (2013) found that administering aspirin-triggered lipoxin A4 to 12-month-old Tg2576 mice, which harbor the Swedish double mutation in APP, activated microglia to an M2 phenotype, resulting in improved...
phagocytic functioning and increased Aβ clearance, as well as improved performance on the water maze.

The conformation of the Aβ can also influence the brain’s immune response. While insoluble fibrillar Aβ_{42} can activate phagocytosis (Weldon et al., 1998), soluble oligomeric Aβ_{40} impairs the phagocytic functioning of microglia in vitro and inhibits ability to clear the Aβ fibrils (Pan et al., 2011). The amount of MHCII positive microglia in the brains of patients and high pathology controls is positively correlated with soluble Aβ in the neocortex, but not fibrillar Aβ_{42} (Lue et al., 1996). Insoluble Aβ_{42} is found in neuritic plaques, but it does not correlate with levels of synaptophysin, which indicates synapse and neuron loss (Lue et al., 1999). In contrast, Aβ_{40} is correlated with synapse loss and can distinguish between demented individuals and high pathology controls (Lue et al., 1999). These findings suggest that it could be the inflammatory response to soluble Aβ, rather than the fully formed amyloid plaques themselves, that may be responsible for driving the neurodegeneration in AD. This process could be driven by the release of cytotoxic substances from M1 microglia and macrophages. In vitro, the induced suppression of fibrillar Aβ phagocytosis can be blocked by incubation with anti-inflammatory cytokines, COX inhibitors, and ibuprofen (Koenigsknecht-Talboo & Landreth, 2005), providing some support for beneficial effects of anti-inflammatory treatments for AD.

**NSAIDs and Anti-inflammatory Treatments**

Prior research has explored the use of anti-inflammatory drugs as a therapeutic strategy against AD. This strategy was first suggested when patients with rheumatoid arthritis using chronic anti-inflammatory drugs were observed to have reduced rates of AD pathology (McGeer, McGeer, Rogers, & Sibley, 1990). Since then, several epidemiological studies have linked long-
term NSAID use to reduced risk of developing AD. A population based prospective study in Rotterdam demonstrated that usage of NSAIDs was linked with a reduction in risk of AD and that the relative risk declined as duration of NSAIDs increased (In t’Veld et al., 2001). In transgenic animal models, chronic NSAID administration has been somewhat effective in preventing or delaying the onset of amyloid deposition, dystrophic neurite formation and inflammation (Lim et al., 2000).

Despite promising epidemiological data and findings with transgenic animals, randomized clinical trials have not provided support for the therapeutic effectiveness of NSAIDs against AD. Studies of patients with established AD have failed to demonstrate a protective effect of various NSAIDs on either cognitive or functional decline (Scharf, Mander, Ugoni, Vajda, & Christophidis, 1999; Aisen et al., 2003). Early results of ADAPT, a large scale longitudinal study, revealed no significant reduction in risk of developing AD after treatment with either celecoxib or naproxen (ADAPT Research Group, 2007), both of which inhibit COX.

The discrepancy in results suggests that the relationship between anti-inflammatory drugs and AD is more complex than once thought. Factors such as age and phase of disease can all play a role. In a study using older cohorts, NSAID treatment actually increased incidence of AD (Breitner et al., 2009). This finding was interpreted with the hypothesis that NSAID use delays AD onset and therefore will lead to a lower prevalence in younger cohorts but an increased frequency in older cohorts. Leoutsakos and colleagues (2011) showed that the effects of NSAID treatment in pre-clinical AD were different depending on whether the patients had a slow or rapid rate of decline, suggesting that the role of inflammation may vary by phase of the disease. It is also possible that a more incisive approach to anti-inflammation is needed. Some components of the inflammatory response could be beneficial for inducing plaque clearance,
while others could by cytotoxic, resulting in neuronal loss. This potential subtlety suggests the need to more deeply understand the roles of individual inflammatory mediators like IL-1 in the pathogenic process.

**Human Gene Profiling Reveals Gene Linkage for Interleukin-1 and AD**

Genetic studies have associated risk for developing AD with certain polymorphisms in genes for several inflammatory agents, with the strongest link involving IL-1α and IL-1β (McGeer & McGeer, 2001). Several studies have shown a correlation between the IL-1α -889 regulatory region and risk of AD. Grimaldi et al. (2000) found that carrying the TT genotype of this polymorphism, which exists in C and T forms, doubles the likelihood of developing AD. This TT genotype was also strongly associated with an earlier age of onset, with carriers of the genotype showing a mean age of onset 9 years earlier than those with the CC genotype. Nicoll et al. (2000) examined a large population of people from centers in the UK and US and found that 12.9% of confirmed AD patients had a TT genotype for IL-1α, compared to only 6.6% of healthy controls. A recent meta-analysis based on 32 case-control studies found a significant link between the homozygous -889T allele and susceptibility to AD, with an odds ratio of 1.2 (Qin et al., 2012).

Two polymorphisms in the gene for IL-1β have also been associated with risk of AD. Homozygosity for the T allele of the +3953 polymorphism, which increases IL-1β production by 4 times compared to a C/C allele, was found to occur in 7.3% in AD patients compared to 4.8% in healthy controls (Nicoll et al., 2000). Individuals who carried homozygous T alleles for both IL1A -889 and IL1β +3953 were 10.8 times more likely to develop AD (Nicoll et al., 2000). A meta-analysis of 8 studies found a significant correlation between the IL-1β +3953
polymorphism and AD, with carriers of the TT genotype about 1.6 times more likely to have the
disease than subjects with CT or CC genotypes (Di Bona et al., 2008).

Grimaldi et al. found a higher risk of late-onset AD in people with a T/T genotype for the
-511 polymorphism of the gene for IL-1β (Grimaldi et al., 2000). However, a meta-analysis
based on 16 studies did not reveal a significantly greater prevalence of the T/T genotype of IL-1β
-511 in AD patients than controls, except when analyzing only Caucasian studies with the
highest statistical power (Di Bona et al., 2008).

**Interleukin-1 and Normal Function/Memory**

There has been substantial evidence linking pro-inflammatory cytokines to impairments
in learning and memory processes. Hippocampal long-term potentiation (LTP), which is critical
for memory consolidation, is impaired by brain elevations of IL-1β in CA1 (Bellinger,
Madamba, & Siggins, 1993), CA3 (Katsuki et al., 1990), and the dentate gyrus (Cunningham,
Murray, O’Neill, Lynch, & O’Connor, 1996). Administration of IL-1β directly to CA1 region of
the rat hippocampus in vitro altered synaptic potentials and inhibited LTP (Bellinger et al.,
1993). Recombinant human IL-1β, when added to the mossy fiber CA3 pathway of mouse
hippocampus in vitro, inhibited LTP even in very low concentrations (2.9pM) (Katsuki et al.,
1990). Pre-treatment of rat hippocampal slices with IL-1β inhibited induction of LTP in the
dentate gyrus, whereas co-application of IL-1ra eliminated the effect (Cunningham et al., 1996).

The disruption of LTP by IL-1β has been demonstrated in vivo as well. In rats,
intraventricular injection of IL-1β significantly increased IL-1β levels in the dentate gyrus and
significantly decreased ability to sustain LTP (Murray & Lynch, 1998). The peritoneal
administration of LPS in rats increased IL-1β expression in the hippocampus and inhibited LTP
in perforant path granule cell synapses (Vereker, O’Donnell, & Lynch, 2000b). The decreased LTP was also accompanied by increases in reactive oxygen species, lowered glutamate release, and cell degeneration in the hippocampus and entorhinal cortex. Although these changes are correlative, they can be blocked by concurrent inhibition of caspase-1, further implicating IL-1β as the mediator of these degenerative effects (Vereker, Campbell, Roche, McEntee, & Lynch 2000a).

Studies using behavioral tests on rodents have demonstrated impaired memory processes in conjunction with increased IL-1β expression in the rodent hippocampus. In mice, administration of exogenous IL-1, LPS or live bacteria impairs spatial learning and retention on the Morris Water Maze (Gibertini, Newton, Friedman, & Klein, 1995; Arai, Matsuki, Ikegaya, & Nishiyama, 2001). Injection of LPS to the rat hippocampus also impairs learning and memory on a passive avoidance test (Tanaka et al., 2006). When LPS is injected with an interleukin-1β inhibitor, memory impairments are attenuated (Tanaka et al., 2011). Blocking the effects of IL-1 through administration of the IL-1 receptor antagonist resulted in facilitation of both short and long term memory in a fear-motivated learning task in rats (Depino et al., 2004). In one study, peripheral administration of LPS disrupted learning after one day of training but not after 4 days of training when learning had plateaued, suggesting that the impaired performance is a result of impaired memory consolidation, rather than sickness behavior (Sparkman, Kohman, Garcia, & Boehm, 2005).

The memory impairments induced by IL-1β are affected by age, with old mice more affected than younger mice. One study showed performance impairments on hippocampal-dependent fear memory tests in aged, but not young, rats after E. coli-induced increases in brain IL-1β levels (Barrientos et al., 2006). In contrast, their performance on a hippocampal-
independent memory task was unaffected. Similarly, intraperitoneal injection of LPS after training on an active avoidance-learning task disrupted memory consolidation for old mice compared to young mice (Tarr et al., 2011). The impaired performance persisted after sickness behavior had dissipated, as measured by escape/avoidance latencies and motor behavior.

Despite the abundance of research showing the detrimental effects of IL-1β overexpression, there is also evidence that IL-1 signaling is necessary for normal hippocampal memory functioning. IL-1β gene expression is increased in the hippocampus during LTP, and blocking IL-1 receptors using IL-1ra impairs the maintenance of the LTP (Schneider et al., 1998). Low doses of IL-1β can actually improve performance on avoidance memory tests (Brennan, Beck, & Servatius, 2003). When IL-1 expression is eliminated, as in IL-1R1 knockout mice, the result is impaired LTP and hippocampal memory formation (Avital et al., 2003). As a result of these findings, Goshen and colleagues (2007) proposed that the effect of IL-1 on memory follows an inverted U-shaped curve. In other words, a certain amount of IL-1 signaling is important for memory, but deviations from that amount in either direction cause impairments. They provided support for this model, showing that intracerebroventricular injections of either IL-1ra or high doses of IL-1β impaired performance on a contextual fear conditioning test, but small doses of IL-1β actually improved performance (Goshen et al., 2007).

IL-1 and Other Behavioral Symptoms of AD

Depression. There is also evidence that IL-1 may impact some of the non-cognitive symptoms of AD. IL-1 induces sickness behavior, which bears many similarities to depression, including withdrawal from the physical and social environment, decreased motor activity, malaise, anhedonia and decreased food intake (Dantzer, O’Connor, Freund, Johnson, & Kelley,
2008). The similarities between these behavioral profiles suggest that cytokine-induced depression represents a maladaptive form of sickness behavior. However, despite the overlap between the two conditions, cytokines can cause depressive symptoms beyond sickness-related behaviors. In patients receiving cytokine immunotherapy, sickness behavior symptoms are universal at first, but only about one third of those patients develop major depressive episodes (Capuron, Ravaud, & Dantzer, 2000). Treatment with antidepressants can prevent the depressed mood and anhedonia associated with the cytokine therapy, but has no effect on fatigue and anorexia (Capuron et al., 2002). In an animal study, mice treated with LPS showed depressive-like behaviors on a forced swim test and a tail suspension test at 24 hours, after sickness-induced motor suppression had returned to normal (Frenois et al., 2007). The same LPS-treated mice showed decreased preference for sucrose solutions despite normal food and water consumption. These results demonstrate a dissociation between depression and sickness-related behaviors despite the overlap between the two.

Several actions of IL-1β have been used to explain the associated depressive symptomatology. IL-1β activates serotonin transporters, increasing serotonin reuptake in synaptosomes from mouse midbrain and striatum (Zhu et al., 2006). Also in mice, LPS induces delayed cellular activity in the limbic system, corresponding to the development of depressive-like behaviors (Frenois et al., 2007). IL-1 activation of the HPA axis may also contribute to the resulting depression. Over activity of the HPA axis is observed in patients with depression, characterized by increased levels of corticotropin-releasing hormone in the brain (Pariante, 2003).

**Anxiety and the Stress Response.** Alzheimer’s patients also commonly exhibit symptoms of anxiety and agitation (Porter et al., 2003; Mega et al., 1996), which may be
associated with inflammation and IL-1 elevations. As mentioned previously, IL-1 is an integral component of the body’s stress response mechanism, mediated by the hypothalamic pituitary adrenal (HPA) axis. In both acute and chronic stressful conditions, the HPA becomes activated resulting in a cascade of events. First, the hypothalamus releases corticotropin-releasing factor (CRF), which triggers the pituitary gland to release adrenocorticotrophic hormone (ACTH), which then travels via the blood stream to the adrenal cortices, stimulating the release of glucocorticoid hormones. These glucocorticoids then travel back to the hypothalamus and pituitary to suppress ACTH production, thus completing the negative feedback loop that regulates the acute stress response (Laugero 2001). Prolonged or severe stress and HPA activation can be harmful. Several studies have showed that stress along with expression of glucocorticoids (cortisol) is associated with atrophy in the hippocampus of both humans and animals, along with deficits in memory, inhibition of neurogenesis, and decreased levels of certain growth factors (see Bremner, 2006 for review).

Research with rodent models has helped demonstrated the link between cytokines and anxiety. Mice injected with IL-1 show HPA activation along with increases in noradrenaline and serotonin, which are both implicated in anxiety and depression (Dunn, 2000). Behavioral tests of anxiety, such as the elevated-plus maze and the light-dark preference test, tend to focus on approach and exploratory drives, particularly in novel settings. Animals exhibiting more anxiety will avoid the exposed arms of the elevated plus maze and avoid the light portion of the light-dark apparatus. These tests have been validated by the alteration of responses following introduction of anxiolytic medications (Belzung & Griebel, 2001). Swiergiel and Dunn (2007) demonstrated that administering either IL-1beta or LPS induced dose-dependent decreases both in open arm entries and time spent in the open arms on the elevated plus test, reflecting increased
anxiety. In contrast, mice with decreased IL-1 activity, due to transgenic overexpression of the IL-1 receptor antagonist, show reduced anxiety both the elevated plus (Oprica et al., 2005) and the light-dark tests behavioral tests (Spulber & Schultzberg, 2010).

In non-clinical and clinical human populations, there are correlations between proinflammatory cytokines and stress/anxiety. Maes et al., (1998) found that medical students expressed elevated levels of proinflammatory cytokines the day before an exam with higher levels of self-reported anxiety corresponding to the higher levels of cytokines. Patients with combat-related post-traumatic stress disorder (PTSD) were shown to have higher IL-β serum levels correlating with the duration of anxiety symptoms (Spivak et al., 1997). Taken together with the animal research, these studies suggest a complex relationship between cytokines, the stress response, and anxiety.

**IL-1 and Other Neurodegenerative Diseases**

Aside from AD, IL-1 has been associated with other neurodegenerative disease. In Parkinson’s disease, elevated levels of IL-1 elevations are reported in nigrostriatal dopaminergic regions (Mogi et al. 1994). Further, Tanaka et al. (2013) found that injecting LPS into the substantia nigra of mice resulted in microglial activation, IL-1β and TNFα production, as well as motor deficits, cell death, and aggregation of α-synuclein. These changes were not seen in IL-1R1 knockout mice. IL-1 is also implicated in multiple sclerosis (MS), a chronic inflammatory, autoimmune, demyelinating disease of the CNS, which is associated with cognitive impairment in about half of patients (Nistico et al., 2014). MS patients have increased IL-1β in peripheral blood mononuclear cells (Heidary et al., 2014) and in CSF, with CSF levels of IL-1β at clinical onset correlating with cortical lesion load and cortical thickness (Seppi et al., 2014). In rats with
experimental autoimmune encephalomyelitis (EAE), an experimental model of MS, IL-1β is expressed by macrophages and microglia in both white and gray matter at sites of early lesion formation (Prins et al., 2013). In amyotrophic lateral sclerosis, increased IL-1β levels are found in the CSF and spinal cord of ALS patients as well as SOD1 mutant mice, an animal model of the disease (van der Meer and Simon, 2010). Blocking IL-1 by inhibiting caspase-1 slows the disease progression (Friedlander et al., 1997). Finally, IL-1 has also been linked to other brain diseases including Creutzfeldt-Jakob disease (Van Everbroeck et al., 2002) and HIV-1 encephalitis (Zhao et al., 2001).

**IL-1 as a Key Player in Alzheimer's-like Cognitive Deficits**

Taken together, the studies presented in this review make a strong case for IL-1 as a key player in Alzheimer’s-like cognitive deficits. However, the inflammatory component of AD is complicated and a clear view of IL-1’s exact role in pathogenesis is still developing. The mixed effectiveness of anti-inflammatory treatment highlights the complexity of the role of inflammation in AD. Potential therapeutic strategies may need to target individual components of the inflammatory response, such as IL-1. If chronic overexpression of IL-1 is responsible for driving the AD pathology and its associated cognitive impairments, chronic inhibition of this cytokine could be beneficial in treating the disease. Many studies described above have used IL-1ra to block the effects of IL-1 in the short term. Another pharmaceutical strategy is the use of an immunoadhesin or trap reagent. One such reagent is the IL-1 Trap or rilonacept (Regeneron Pharmaceuticals). The IL-1 Trap consists of a forced IL-1 receptor 1 homodimer fused to a mouse Fc fragment. This trap binds IL-1 at a high affinity, preventing it from binding to its endogenous receptor, and therefore serves as an antagonist of IL-1 signaling (Economides et al.,
2003). Using transgenic mice that overexpress mutated pathogenic APP, one could start treatment at the point when symptoms and pathology usually become evident to determine whether IL-1 inhibition would slow and/or reverse disease progression. In order to have the most impact, treatment should begin early in the disease course. Although starting treatment much earlier may be more effective at preventing the pathological signs of the disease from developing, is not as relevant to clinical populations, as people usually do not start treatment for Alzheimer’s Disease before any symptoms develop. Therefore, the best approach would be to initiate treatment at time points when symptoms have appeared. After a period of chronic treatment with this drug, memory could be assessed to look for cognitive improvement using behavioral tests such as the Morris Water Maze. It would also be important to assess the effect of chronic IL-1 inhibition on the deposition of amyloid plaques, as well as on microglial activation and investment. This research would help to further elucidate any causal relationship between IL-1, neuroinflammation, and AD pathology.

The following specific aims are proposed to address the role of IL-1 signaling in the behavioral and anatomical pathology of Alzheimer’s-like disease:

Specific Aim 1: To examine the effect of chronic systemic inhibition of IL-1 signaling on learning and memory in AD model transgenic mice and wild type controls.

Specific Aim 2: To determine whether chronic systemic inhibition of IL-1 signaling affects amyloid plaque pathology in the brains of AD model transgenic mice.
Specific Aim 3: To determine whether chronic systemic inhibition of IL-1 signaling affects microglial density and size in the hippocampus of AD model transgenic mice, both in general and surrounding amyloid plaques.

Specific Aim 4: To determine whether quantitative measures of amyloid plaque pathology and microglial measures are predictive of performance on measures of learning and behavior in AD transgenic model mice, and whether chronic inhibition of IL-1 signaling alters this relationship.
Chapter 2

GENERAL METHODS

Subjects

Subjects were 40 male mice run in two cohorts of 20 mice each. The transgenic mice used in the first cohort were on a C57Bl/6 background (The Jackson Laboratories, Stock number = 005866). Due to problems with availability of this strain, the mice used in the second cohort were from a slightly different background lineage (Jackson Laboratories, Stock number = 004462). C57Bl/6 mice were used as the recommended wild type controls (Stock number = 000664). Of each cohort, ten of the mice were wild type (WT) animals and ten were tandem transgenic (Tg) for both the Swedish pedigree mutation in the amyloid precursor protein (Mo/HuAPP695swe) and the high Alzheimer’s risk polymorphism in presenilin-1 (PS1-dE9).

The mice were housed either singly or in small groups of three or fewer animals per cage. All animals were initially housed in small groups and only separated in the case of fighting. Due to concerns that isolation can have significant effects on animal behavior, a log linear analysis was conducted to make sure that the number of singly housed animals was evenly distributed across groups. The results of this analysis revealed no difference between genotypes and treatment groups ($G^2(4)=3.2, p=0.52$). Animals were housed in a temperature-stabilized facility on a 12:12 light:dark cycle (lights on 07:00), with food and water available ad libitum. All procedures were conducted with approval from and in strict compliance with the animal welfare policies of the Institutional Animal Care and Use Committee of Queens College of the City University of New York.
Experimental Design and Timeline

Animals were received from Jackson Laboratories at about 12 weeks of age. They were introduced to their home cages and allowed to acclimate and age until behavioral testing was initiated. Animals were given the open field test at about 5 months of age to assess for baseline locomotor activity. At about 8 months of age, when previous studies have reported the emergence of behavioral and anatomical abnormalities, injections began and continued for 5 months. During the fifth month of treatment, behavioral testing was conducted to evaluate learning and memory as well as general locomotor activity and anxiety. After the completion of behavioral testing, animals were perfused and their brains were collected and prepared for further analysis.

Proteins and Injections

In order to inhibit interleukin signaling, animals were administered either mouse IL-1 Trap, which consisted of a forced homodimer of the extracellular binding domain of IL-1 receptor 1 fused to mouse Fc fragments, or the protein control mFc (a generous gift from Regeneron Pharmaceuticals, Tarrytown, NY, USA) starting at about 8 months of age. Both the mIL-1 Trap and mFc were kept frozen (at -80°C) until use and were then thawed and diluted in sterile phosphate-buffered solution (PBS) (Sigma-Aldrich, St. Louis, MO, USA). Within each cohort, five TG animals and five WT animals received mouse IL-1 Trap (mIL-1 Trap) at a dose of 10 mg/kg. The rest of the mice received mouse Fc (mFc) at the same dose and volume to control for protein load (Table 1). Injections were administered subcutaneously twice per week. Mice were weighed weekly in order to establish dosage and evaluate animal health. The injections continued for 5 months.
Because the IL-1 trap was administered peripherally in this experiment, it was not known how much, if any, of the compound would reach the brain. The mIL-1 trap is a large protein and therefore is not likely to cross over the blood brain barrier. The purpose of this study was to assess the clinical efficacy of the drug in AD-like pathology and therefore, the exact mechanism by which peripheral IL-1 inhibition has its effect was not the main focus of our experiments. However, if peripheral mIL-1 Trap administration has no measurable effects on either behavior or pathology, it would raise the question of whether the drug has no effect, or whether it simply failed to reach its target. In that case, further work would need to be done to differentiate between these possibilities.

**Tissue Collection and Processing**

After 5 months of treatment, the animals were sacrificed and brains were prepared for histological analysis. Twenty-four hours after their last mFc or mIL-1 Trap injection, all animals were overdosed with a pentobarbital-based euthanasia solution. Cold heparinized isotonic (0.9%) saline was perfused transcardially to exsanguinate the animal, followed by perfusion with 4% paraformaldehyde first in acetate, and then borate buffer (100ml each). Upon completion of the perfusion, brains were removed and placed in 30% buffered sucrose for 3-7 days. The fixed brains were then sectioned coronally at 50µm using a sliding microtome (American Optical Company, Buffalo, New York). Sections were placed into a 24-well plate and stored in an ethylene glycol based cryoprotectant solution (Watson et al., 1986) at -20°C until they were stained.
Table 1. Experimental Design

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Chapter 3

BEHAVIORAL TESTING

The most prominent neuropsychological deficit in AD patients is impaired verbal and spatial memory. Animal models of the disease also demonstrate impairments in hippocampally-mediated memory tasks such as spatial mazes (Hall and Roberson, 2012). Since memory is the first and most prominent functional impairment in AD, the most important question for any treatment is whether it can slow or prevent this functional memory decline. There is growing evidence that implicates IL-1 as a contributor to AD pathology (Shaftel et al., 2008). In addition, elevated IL-1 levels have been linked to impairments in hippocampal long-term potentiation (Bellinger et al., 1993; Katsuki et al., 1990; Cunningham et al., 1996) as well as impaired performance on the Morris Water Maze (Gibertini, Newton, Friedman, & Klein, 1995; Arai, Matsuki, Ikegaya, & Nishiyama, 2001). In contrast, blocking IL-1 protects against memory impairments on other hippocampal-dependent tests (Tanaka et al., 2011). Therefore, we hypothesized that long term inhibition of IL-1 using the mIL-1 Trap in transgenic mice would preserve learning and memory, as assessed by the Morris Water Maze.

In addition to learning and memory, we assessed general locomotor activity. Alzheimer’s transgenic mice have shown alterations in general locomotor and exploratory behaviors (Lalonde, Fukuchi, & Strazielle, 2012). Because of IL-1’s role in sickness-related behaviors, including decreased motor activity, withdrawal from the physical and social environment, and general malaise (Dantzer, O’Connor, Freund, Johnson, & Kelley, 2008), it is possible that long term IL-1 inhibition could alter exploratory and locomotor activity as measured on the Open Field test. It was especially important to assess for Open Field activity as a control for memory
performance, because differences in activity level can influence water maze performance.

Finally, the elevated plus maze and light dark test were conducted as measures of anxiety-like behaviors.

**Methods.**

**Morris Water Maze.** The Morris Water Maze Test was conducted in order to evaluate spatial learning and memory. A pool measuring 105 cm in diameter and 35 cm in depth was filled with water made opaque with non-toxic white paint. The pool was then divided conceptually into four quadrants, one of which contained an escape platform hidden 2.5 cm beneath the surface. Animals were placed in a different, pseudo-randomly selected quadrant at the start of each trial, and the latency to escape the maze onto the hidden platform was measured. There were two trial blocks per day for five days, each consisting of three one-minute trials. Between trials, animals were held with a towel for approximately 30 seconds before being placed back in the water. After each block, they were placed in a holding cage lined with towels until dry and then returned to their home cages. The testing was conducted at the same time each day with a 3-hour interval between the first and second daily blocks. Any animal not locating the platform within one minute was assigned a latency of 60 seconds and guided to the platform by hand before being removed from the water. Normal animals are expected to demonstrate a decrease in latency to escape across trials in the water maze, indicating acquisition of the location of the platform over time.

One hour after the final block of water maze acquisition testing, animals were returned to the maze for a 30 second probe trial with the platform removed to test their retention of the platform’s location. Retention was assessed by measuring the amount of time animals spent in
the quadrant that formerly housed the platform, termed the “goal quadrant.” The number of times the animals swam over the spot where the platform was located was also counted. Animals with normal retention are expected to spend more time in the “goal quadrant” than in other quadrants and to make more “platform crosses”, thus demonstrating retention of the spatial location of the escape platform.

For each trial block, the animals’ median latency to escape the maze across the three trials was recorded and these median latencies were used for statistical analysis. In addition, animals’ swim speeds were estimated by dividing latency to escape by the number of maze quadrants crossed per trial to calculate mean quadrant crossing time as a surrogate for swim speed. The median quadrant crossing time for each trial block was used for statistical analysis.

**Open Field.** The Open Field test was conducted in order to assess basic locomotor activity. Animals were placed into a white, box-like apparatus measuring 48 wide x 48 long x 24 high cm with the inside floor divided into nine grids, each measuring 16 x 16 cm. Animals were placed in the center grid and allowed to freely explore the apparatus for a six-minute trial. An animal was considered to have crossed from one grid to another when at least three paws crossed over the grid line. The number of total grid crossings was recorded as a measure of general locomotor and exploratory behavior. Rearing behaviors, defined as any time an animal stood up on its hind legs, were recorded as another measure of exploratory activity.

**Elevated Plus Maze.** The elevated plus maze test was conducted to assess anxiety-like behaviors. The apparatus was composed of a platform measuring 15 x 15 cm elevated 114 cm on a central pole. Four arms were joined to the platform, each measuring 61 x 15 cm, arranged
in a plus-shaped configuration and equal in height relative to the platform. Two of the arms were white, open platforms oriented directly across from each other. Perpendicular to the open arms were two black arms enclosed by walls measuring 30 cm high. Animals were placed on the platform and allowed to freely explore the maze for five minutes. The number of seconds the animals spent on the platform and in the open and closed arms was recorded. Increased time spent in the closed arms is traditionally considered indicative of heightened anxiety, as rodents tend to avoid open, brightly lit areas that are unfamiliar or might expose them to danger from airborne predators. The total number of arm entries was also recorded as a measure of locomotor activity as well as the proportion of entries made to the closed arm as a second measure of anxiety-like behavior. This test was conducted with all animals two weeks before the first injection to establish a baseline before treatment. The test was conducted again 2 weeks prior to sacrifice, after almost 5 months of treatment.

**Light-Dark Exploration.** The light-dark exploration test was conducted as an additional measure of anxiety-like behavior. Animals were placed in a rectangular apparatus measuring 45 long x 20 wide x 20 cm high, half of which was white and open at the top (the “light” side), and half of which was black and covered (the “dark” side). Animals were placed in the center of the light side and allowed to freely explore the apparatus for five minutes. The amount of time animals spent in the light and dark sides, respectively, was recorded. As in the elevated plus maze, increased time spent in the dark, enclosed portion of the apparatus is considered indicative of increased anxiety.
**Statistical Analysis.** For water maze acquisition, the median latency to escape onto the goal platform was recorded for each animal for each trial block. A three-way mixed Factorial analysis of variance (ANOVA) was conducted using treatment and genotype as the between groups independent variables and trial block as a repeated measure variable. For retention, the proportion of time spent in the goal quadrant was recorded for each animal along with the number of platform crosses. Two-way ANOVAs (genotype x treatment) were conducted with each of these as dependent variables.

To evaluate locomotor activity and exploratory behavior, the number of grid crossings in the open field test was counted for each animal. The number of times an animal engaged in rearing behavior was also recorded. A two-way ANOVA (genotype x treatment) was conducted using the number of total grid crossings as well as rears.

The elevated plus and light dark tests were both conducted as measures of anxiety. For the elevated plus, the proportion of time spent in the closed arm was recorded for each animal. In addition, number of total arm entries was recorded as a measure of overall exploratory activity and the proportion of those entries made to the closed arm was calculated as another measure of anxiety. Two-way ANOVAs (genotype x treatment) were conducted for these dependent variables. For the light dark test, the proportion of time spent in the dark side was recorded and two-way ANOVA (genotype x treatment) was conducted.

For each measure, the analyses were run first using cohort as a factor in order to assess for cohort effects. This was especially important because the background strains used between the first and second cohort were slightly different, raising concerns that the treatment could have affected the cohorts differently. If there were no interactions between cohort and treatment, the data were collapsed across cohorts.
**Results**

**General Health.** Mice were weighed and evaluated weekly to assess their general health. Out of the original 40 animals, 37 survived to sacrifice. In the first cohort, one transgenic animal in the mFc-treated group died before the behavioral testing stage. From the second cohort, one transgenic animal in the mIL-1 Trap group died before the behavioral testing stage and one wild type animal in the mIL-1 Trap group died after completing some behavioral testing. At the final weighing before perfusion, the transgenic mice weighed about 27% less than the wild type mice (F(1,33)=55.46, p<.001) (*Figure 2*). Although the transgenic mice weighed less, they were actually closer to the normal body weight for adult male mice (Mazzaccara et al., 2008), and appeared grossly healthy. The mIL-1 Trap had no negative impact on body weights (F(1,33) =0.00, p=.994) and the mice appeared grossly healthy throughout the study.

![Figure 2. Average weight of each group throughout the treatment.](image-url)
**Water Maze.** A three-way mixed Factorial ANOVA (genotype x treatment x trial block) showed that the wild type animals performed significantly better overall than the swAPP/PS-1 double transgenic animals in water maze acquisition (F(1,33)=29.71, p<.001), as expected based on previously published data. The mIL-1 Trap had no significant overall effect on water maze performance (F(1,33)=2.76, p=0.11), but there was a significant interaction between the two factors (F(1,33)=4.61, p=.039; Figure 3), with the Trap selectively improving performance in the transgenic animals. An analysis of cohort effects revealed a significant cohort by genotype interaction, such that the transgenic animals of the second cohort performed worse overall than those in the first cohort. However, there was no cohort by treatment effect and no cohort by genotype by treatment effect, indicating that the Trap affected the mice similarly across both cohorts.

We also assessed whether differences in swim speed could be influencing our findings by evaluating mean quadrant crossing time for the animals. Slower swim speeds have been reported in transgenic AD mice (Ying et al., 2010), which can interfere with an animal’s latency to swim to the platform. A three-way mixed Factorial ANOVA (genotype x treatment x trial block) revealed no significant differences in quadrant crossing time across genotype (F(1,33)=.61, p=.442) or treatment (F(1,33)=1.13, p=.296), and no genotype by treatment interaction (F(1,33)=.47, p=.50). In addition, we did not see any significant general locomotor differences in the open field test (see Open Field section), suggesting that motor differences did not account for the acquisition results observed.

On the retention portion of the water maze, wild type animals spent significantly more time in the goal quadrant (F(1,33)=18.83, p<.001) and made significantly more platform crosses
(F(1,33)=8.86, \( p=0.005 \)) than transgenic animals, confirming the previously-reported memory impairment in the Alzheimer’s model transgenics. There was no significant effect of mIL-1 Trap treatment on proportion of time spent in the goal quadrant (F(1,33)=.313, \( p=.58 \)) and there was no interaction between the factors (F(1,33)=.04, \( p=.84 \)) (Figure 4). There was also no significant effect of treatment on number of platform crosses (F(1,33)=2.29, \( p=.14 \)) and no interaction between treatment and genotype (F(1,33)=1.63, \( p=.21 \)) (Figure 5). An additional two-way ANOVA was performed to look at escape times in the final block of water maze acquisition (block 10) alone to see how the animals differed shortly before the retention phase. At this point, the transgenic animals showed a statistical trend toward longer latencies to escape to the platform compared to wild type animals (F(1,33)=3.018, \( p=.092 \)). However, there was no significant overall effect of treatment (F(1,33)=2.689, \( p=.111 \)) and no interaction between genotype and treatment (F(1,33)=.122, \( p=.729 \)) at this point. This additional analysis suggests that by the end of water maze acquisition, the animals had all eventually learned the maze to a similar level.
**Water Maze Acquisition**

*Figure 3. Water Maze Acquisition.* Wild type animals performed significantly better overall than transgenic animals in water maze acquisition (F(1,33)=29.71, p<.001). There was a significant interaction between genotype and treatment (F(1,33)=4.61, p=.039), such that the mIL-1 Trap improved acquisition performance for transgenic animals.

**Water Maze Retention (time in goal quadrant)**

*Figure 4. Water Maze Retention (time in goal quadrant).* Wild type mice spent significantly more time in the goal quadrant than transgenic animals. However, there was no significant effect of treatment and there was no interaction between the factors. ***p<.001
Figure 5. Water Maze Retention (number of platform crosses). Wild type mice made significantly more platform crosses than transgenic animals. There was no significant effect of treatment on number of platform crosses and no interaction between factors.

To further explore differences in the learning process between these groups, an additional set of water maze analyses were conducted. First, different scores were obtained for all blocks occurring on the same day (eg. block 2 minus block 1), yielding 5 within-day difference scores. Then, difference scores were obtained using the first block of one day and the last block of the day before (eg. block 3 minus block 2), yielding 4 difference scores. This was done to look at differences in memory consolidation by assessing learning that occurred overnight. Difference scores for both within-day and overnight were compared for both genotypes and treatment groups across all days of the Water Maze test. Data were analyzed using a mixed factorial ANOVA with difference scores across test days as the within-subjects factor and genotype and treatment as between-groups factors.

Regarding within-day analyses, no differences were found overall in difference scores across the 5 days. That is, the extent to which the animals’ scores changed from the first to
second block of the day did not differ throughout the course of the 5-day test (F(4,132)=.500, p=.736). There were also no between-groups differences between genotype (F(1,33)=.429, p=.517) or treatment group (F(1,33)=1.429, p=.241), and no interaction (F(1,33)=1.425, p=.241).

When the overnight difference scores were analyzed, a significant effect of test day was found (F(3,99)=3.518, p=.018) Overall, the difference scores were negative for day 1-2 (indicating learning) and became more positive as the test went on, suggesting that there was learning occurring overnight early in the experiment and that the overnight boost lessened as the test progressed. There was also a significant effect of treatment, such that animals treated with mIL-1 Trap had significantly lower overnight difference scores (indicating better learning) than animals treated with mFc (F(1,33)=5.353, p=.027). This suggests that treatment with the trap improved memory consolidation overnight. Interestingly, there was no significant effect of genotype on this measure (F(1,33)=.210, p=.650) and no interactions with genotype and treatment (F(1,33)=.138, p=.713).

**Open Field.** In order to examine baseline differences by genotype in locomotor activity and exploratory behavior, the number of overall grid crossings was compared between wild type and transgenic animals at 4-5 months old. A t-test revealed that transgenic mice made significantly fewer grid crosses than wild type animals (t(38) = 3.18, p=.003) (Figure 6a) Transgenic mice also made significantly fewer rears than wild type animals (t(38) = 3.512, p=.001) (Figure 6b). This suggests that swAPP-PS1 mice show a reduction in general locomotor activity and exploratory behavior at 4-5 months of age.
Figure 6. Open field results for 4-5 old animals. 4-5 month old transgenic animals made significantly fewer grid crosses (a) and significantly fewer rears (b) on the Open Field test relative to wild type controls. **p<.01, **p ≤.001.

The open field test was conducted again after 4 months of treatment with the mIL-1 Trap at which point the mice were approximately 12 months old. A two-way ANOVA (genotype x treatment) was conducted to examine differences in total number of grid crossings. Overall, there was no difference in total number of grid crossings between wild type and transgenic animals (F(1,33)=.699, p=.409), and no difference between animals treated with mFc and those treated with mIL-1 Trap (F(1,33)=.18, p=.673). There was no interaction between genotype and treatment (F(1,33)=.03, p=.873) (Figure 7a). Whereas younger transgenic mice made fewer rears than wild type controls, as older mice, the transgenics showed a trend towards making more rears than wild types, although this did not reach statistical significance (F(1,34)=3.107, p=.087). There was no overall difference between the two treatment groups (F(1,34)=.011, p=.918) and no genotype by treatment interaction (F(1,34)=.000, p=.984) (Figure 7b). These results suggest that although there were differences seen in locomotor activity as 4-5 month old animals, transgenic mice did not differ significantly in overall level of locomotor activity compared to wild type mice at 8-9 months of age, and that level of activity was not affected by treatment with mIL-1.
Trap. Since the second run of open field was conducted within two weeks prior to water maze testing, these results suggest that differences in water maze performance are not likely to be explained by differences in general activity and/or exploratory behavior. Our finding of no significant differences among the groups in mean quadrant crossing time in the water maze further suggests that locomotion was not a confounding variable in our cognitive tests.

**Figure 7:** Open field results for 8-9 month old animals. A) No differences were found in total number of grid crossings based on genotype or treatment and there was no interaction between genotype and treatment. B) Transgenic mice showed a statistical trend toward making more rears than wild types, but there was no overall difference between the two treatment groups and no genotype by treatment interaction.
**Elevated Plus Maze.** In order to assess anxiety-like behaviors, the elevated plus test was conducted in one cohort of animals, both right before beginning treatment with mIL-1 Trap and approximately one week before perfusion. The proportion of time spent in the closed arms was compared between the two genotypes. A t-test revealed that young transgenic mice spent significantly more time in the closed arms compared to the wild-type animals ($t(18)=4.612, p<.001$) (Figure 8a). This result suggests a heightened level of anxiety in 7-month old transgenic mice compared to their wild type counterparts. The transgenic animals made fewer total arm entries than the wild type animals, suggesting reduced exploratory activity on this test ($t(18)=3.596, p=.002$) (Figure 8b). The transgenic animals also showed a statistical trend towards making a higher proportion of their arm entries into the closed arms ($t(18)=2.018, p=.064$) (Figure 8c).

When the elevated plus test was run 5 months later, there were no longer any differences in the anxiety measures between groups. A two-way ANOVA (genotype x treatment) showed no significant difference in time spent in the closed arms between wild type and transgenic animals ($F(1,14)=.000, p=.994$), no significant effect of treatment ($F(1,14)=1.501, p=.238$) and no significant interaction between genotype and treatment ($F(1,14)=.200, p=.66$) (Figure 9a). When analyzing the data in terms of proportion of closed arm entries, there was also no difference between genotypes ($F(1,14)=.000, p=.987$), no effect of treatment ($F(1,14)=.177, p=.680$) and no genotype by treatment interaction ($F(1,14)=1.157, p=.300$) (Figure 9c). Unlike the Open Field test, however, old animals did show a difference in overall activity on the elevated plus. Transgenic mice at 12 months of age still made significantly fewer overall arm entries than wild type animals ($F(1,14)=6.162, p=.026$). There were no differences between treatment groups ($F(1,14)=1.040, p=.325$) and no interaction ($F(1,14)=1.307, p=.272$) (Figure 9b).
Figure 8. Elevated plus results for 7-month old animals. a) At 7 months of age, transgenic animals spent a significantly greater proportion of time in the closed arms than the wild type animals. b) They made significantly fewer entries overall, but c) showed a statistical trend (p=.064) towards making a higher proportion of those entries to the closed arms.
a) Elevated Plus (12 mos): Proportion of time in closed arms

b) Elevated Plus (12 mos): Total Arm Entries

c) Elevated Plus (12 mos): Proportion of Arm Entries to Closed Arm

**Figure 9. Elevated Plus results for 12-month old animals.** a) At 12 months of age, there were no longer any differences between genotypes in time spent in the closed arms of the apparatus. There was also no effect of treatment with mIL-1 Trap and no interaction between treatment and genotype. B) Transgenic mice made significantly fewer overall arm entries than wild type animals, but there were no differences between treatment groups and no interaction. C) There were no differences in proportion of closed arm entries between genotypes and treatment groups, and no interaction.
**Light-Dark Test.** A second test of anxiety was conducted following the elevated plus maze test in the aged animals only. The proportion of time spent in the dark half of the apparatus was compared using a two-way ANOVA (genotype x treatment). Similar to the elevated plus maze, there were no differences found between wild type and transgenic animals (F(1,16) = .057, p=.815) or between animals treated with mIL-1 Trap vs. mFc (F(1,16)=2.573, p=.128). There was no significant genotype x treatment interaction (F(1,16)=.282, p=.603) (Figure 10).

![Light Dark: Proportion of time in dark side](image)

**Figure 10. Light Dark proportion of time spent in dark side.** At 12 months, there were no significant differences in time spent in the dark half of the apparatus between the transgenic and wild type animals, no affect of treatment, and no genotype by treatment interaction.

**Interim Discussion**

Our findings indicate that the mIL-1 Trap selectively improved water maze performance in the Alzheimer’s model transgenic animals. This pattern of behavioral results suggests that IL-1 inhibition can slow the cognitive decline resulting from overexpression of mutant Presenilin-1 and the Swedish APP mutation without impacting performance in normal mice. The effects of IL-1 inhibition shown here are in agreement with shorter term studies that link sustained overexpression of hippocampal IL-1β in mice with impaired spatial memory, glial activation, and
increased pro-inflammatory cytokine expression (Hein et al., 2010; Moore et al., 2009). A study using a transgenic rat model of Alzheimer’s disease demonstrated improved spatial memory, decreased hippocampal neuronal loss, and reductions of hippocampal IL-1β and TNFa levels in rats treated with Tetrandine (He et al., 2011). In the present study, IL-1 inhibition improved acquisition while having no effect on retention, suggesting that the treated mice may have used compensatory strategies to assist with their performance of the task.

The extra water maze analyses of difference scores from blocks within the same day and overnight suggest that the Trap is boosting memory by affecting memory consolidation. Specifically, animals treated with mIL-1 Trap showed a greater boost in memory from the last trial of one day to the first trial of the next when compared to the mFc treated animals. However, this was only observed as a main effect of treatment that did not interact with genotype. This suggests that the effects of IL-1 inhibition on overnight memory consolidation were beneficial for both transgenic and wild type animals and therefore, it may not explain the selective improvement of trap treated transgenic animals on the overall test. But, if the IL-1 traps memory effects are most evident when looking at memory from one day to the next, it could be that the period between the last acquisition trial and the retention probe were not long enough to see differences, which could explain the lack of effect seen between treatment groups on this probe trial.

One important question that is often ignored in animal studies is how the behavioral testing can be linked to cognitive performance in humans. Specifically, can we draw parallels from the water maze to human cognitive testing? Clinically, a diagnosis of AD is usually based more on verbal tests, which obviously cannot be conducted with rodents. However, spatial disorientation and getting lost is a common symptom of early AD (McShane et al., 1998;
Guariglia and Nitrini, 2009). On objective testing, visuospatial memory deficits are observed in AD patients. Okonkwo et al (2014) reported that baseline performance on the Brief Visuospatial Memory Test-Revised (BVMT-R), which involves learning and delayed memory of visual figures and their locations, was the best predictor of cognitive decline in a cohort of cognitively normal middle-aged adults (age 40-65). In the Baltimore Longitudinal Study of Aging, making more errors on the Benton Visual Retention Test was associated with an increased risk of an AD diagnosis up to 15 years later (Kawas et al., 2003). In addition, a study of non-demented oldest old adults (ages 82-95) demonstrated that immediate and delayed memory of a complex figure (Rey Osterreith) declined significantly more over a 7-9 year period in those with a positive amyloid PET than those with a negative PET scan (Snitz et al., 2013).

Although the aforementioned neuropsychological tests are grouped in the category visuospatial learning and memory, the tasks may differ from navigating and remembering a maze. There have been some attempts to more directly translate these rodent tests to humans and tap into these spatial navigation deficits, using real world and virtual reality paradigms. Astur et al (2002) actually developed a virtual Morris Water Maze task to show that humans with hippocampal resections had spatial navigation impairments relative to those with resections in other brain areas, although this was not conducted in AD patients. Cushman et al. (2008) found significant differences in navigational ability and spatial memory using real world and virtual reality environments such that those with early AD performed worse than early MCI who performed worse than controls.

Even with these more targeted spatial tests, important differences remain. First, the time course of human testing vs. animal testing is different. Even when several learning trials are used, as in the human virtual water maze, the time of the test is compressed relative to animal
testing, which can span several days. Also it may be impossible to completely remove the verbal component from human cognitive testing. In Cushman et al’s (2008) study, delayed verbal memory along with virtual reality performance, was a significant predictor of real world navigational performance. Performance on spatial tests could partially be influenced by verbal deficits influencing the ability to name landmarks or construct a narrative of a visual scene or navigational path. Despite these limitations, it is important to consider the application of any testing with rodent models to human patients.

In addition to learning and memory, past studies have used tests such as the open field to examine changes in locomotor and exploratory activity in transgenic animals. Several studies have shown hyperactivity in Alzheimer’s model transgenic strains, including in the Tg2576 line (Lim et al., 2001) and the swAPP/PS1 strain used in the present study (Cheng et al., 2013). We did not replicate these findings and in fact found a significant pattern of hypoactivity in the younger transgenic mice. However, a thorough look at the literature reveals a more complex relationship between AD mutations and locomotor activity, which may depend on the task parameters, sex of the animals, and the transgenic strain used (Lalonde et al., 2012). Though many studies show open field hyperactivity, others show no locomotor differences. Lalonde, Kim, and Fukuchi (2004) found no difference in open field activity in 7-month-old male and female swAPP/PS1 (delta e9) mice relative to wild type controls. Other studies looking at models with slightly different swAPP and PS1 mutations even found hypoactivity, including Liu et al. (2002) who found reduced open field locomotor activity and rearing in 7 month old male swAPP PS1(A246E) mice. This variability is also reflected in humans with AD, as some may exhibit signs of agitation while others experience apathy and decreased activity (Di Iulio et al.,
2010; Chung & Cummings, 2000). The reasons for this variability are still not completely understood.

Age also appears to be a factor with many transgenic models showing changes in activity level throughout the life span. In the present study, by the time the mice were older (12 months), any differences in open field activity across groups had disappeared. Locomotor activity no longer differed between genotypes. Age-related changes from hypo- to hyperactivity have been reported in APP751SL mice from 2 months to 19-20 months (Blanchard et al., 2009) and from 6 months to 24 months in APP23 mice (Dumont et al., 2004). One study using 10-14 month old male swAPP/PS1(dE9) mice, the exact strain used in this experiment, found no difference in open field locomotor activity or rearing (Hartmann et al., 2010). Since motor activity of normal mice decreases with age (Lalonde et al., 2012), if the predominant phenotype of this transgenic strain is hypoactivity, it would make sense that the differences are more apparent when the mice are young.

Despite previous research showing some support for anti-inflammatory treatment affecting locomotor activity (Lim et al., 2001), we did not find any effect of treatment with mIL-1 Trap on open field activity. Since the primary purpose for including this measure was as a control for learning and memory tests, the main conclusion of these results is that differences in water maze performance between treatment groups cannot be attributed to changes in locomotor activity or exploratory behavior.

In addition to differences in overall locomotor activity between young transgenic and wild type animals, we also found a significant difference on the elevated plus test, which is used as a measure of anxiety. Young (7 month) transgenic animals spent significantly more time in the closed arms of the apparatus than the wild type controls. Increased time in the closed arms is
thought to indicate heightened anxiety since the animals avoid brightly lit areas that could expose them to danger from predators. As with the open field test, the behavior of TG mice in the elevated plus test depends on several factors, including the exact model, sex and age of the mice and parameters of the task. In contrast with our results, Lalonde and colleagues found increases in open arm exploration, suggesting disinhibition, in 7-month-old (2004) and 12-month-old (2005) swAPP/PS1 mice, the same model used in the present study. However, not all studies have revealed the same pattern. Studies using transgenic models with slightly different APP and PS1 mutations than the mice used here showed no difference between transgenic and wild type mice at 6 months (Filali, Lalonde, & Rivest, 2011) and heightened anxiety at 14 months (Puolivali et al., 2002).

The results of the elevated plus also shed light on our young open field findings. The open field test is most often used as a measure of locomotor activity but it involves placing the animals in a novel environment, which can be intrinsically anxiety provoking. Viewed through the lens of increased anxiety, the reduced overall activity and number of rears in young transgenic animals could be viewed as anxious “freezing” behavior.

When the animals were older (12 months old), the elevated plus test no longer revealed any differences between the two genotypes. Wild type and transgenic animals spent a similar amount of time in the closed arms and there was no effect of treatment with the mIL-1 Trap. The light dark test was performed as second test of anxiety in the older animals and also revealed no differences between any groups. It is possible that the heightened levels of anxiety seen in the earlier testing signify a prodromal stage of AD that wanes as the disease progress. It also may be that the animals become acclimated to repeated testing throughout the experiment and no longer experience an anxiety response to these stimuli. In order to fully differentiate between these
possibilities, more detailed behavioral testing would need to be conducted across the life span of these animals. For purposes of this study, the lack of differences in anxiety measures between the older groups further supports our conclusion that improvements on the water maze test are due to improved learning and memory rather than anxiety provoked behavioral arrest.
Chapter 4

AMYLOID PLAQUE ANALYSIS

One of the most prominent pathological features of AD is the presence of amyloid plaques throughout the cerebral cortex. Since the discovery of amyloid β protein in senile plaques, the question of amyloid’s role in AD has driven a large portion of AD research. For decades, studies have focused on the amyloid cascade hypothesis (ACH), stating that deposition of Aβ is the trigger responsible for the pathological and functional changes seen in AD (Hardy and Higgins, 1992; Reitz 2012). Although support for the ACH has waned in recent years, amyloid is still a focus of AD research both in humans and animal models. In fact, many transgenic AD mouse models utilize genes responsible for processing of APP that are linked to familial AD (Citron et al., 1992; Sherrington et al., 1995). Thus, in many transgenic AD mouse models, including the swAPP-PS1 model used here, amyloid plaques are the most obvious pathology.

Because of the importance of Aβ plaques in the brains of these mice, it was important to assess the effect of long term IL-1 inhibition on amyloid plaque deposition. There is evidence that IL-1 may contribute to Aβ plaque pathology by promoting APP synthesis (Goldgaber et al., 1989) and by encouraging the formation of mature plaques from β amyloid aggregates (Griffin et al, 1995). Inducing IL-1 expression with LPS has been shown to increase APP and Aβ accumulation (Sheng et al., 2003, Guo et al., 2002). As shown in the previous chapter, chronic, systemic inhibition of IL-1 using mIL-1 Trap in these transgenic mice resulted in a significant improvement in water maze performance compared to mice not given IL-1 Trap. Our next goal was to try to determine whether this inhibition of IL-1 was also associated with a reduction of
amyloid plaque pathology. If the mIL-1 Trap had decreased the number or size of amyloid plaques in the hippocampus, this could provide an explanation for the improvement in memory functioning.

Methods

**Histology.** Sections were stained in a 1:12 series in Congo Red (Sigma-Aldrich C6767) to detect the presence of amyloid plaques (protocol in Wilcock, Gordon & Morgan, 2006). The sections were first mounted on slides and allowed to air-dry overnight. After rehydrating in distilled water, slides were incubated first in an 80% ethanol solution saturated with NaCl and then in Congo Red solution (0.2% Congo Red in 80% ethanol saturated with NaCl). They were then rinsed in 95% and 100% ethanol, cover-slipped with xylene-based mounting medium and allowed to dry overnight. All excess adhesive was removed from the dry slides using a glass cleaning solution.

**Image Analysis.** Amyloid plaque burden was analyzed by contrast analysis using the Image J image analysis software program (NIH). For each animal, a minimum of 2 bilateral sections of hippocampus were photographed at 10x. However, the method used for capturing images were slightly different from cohort 1 to cohort 2 because of improvements in imaging technology available that occurred between cohorts. For cohort 1, images were captured in 10x using the Picture Frame program. Pictures were taken in one session to minimize differences in illumination between images. For cohort 2, slides were placed in the slide rack compartments (hotels) of an automated imager (Leica, Model SL801). The imager was set to scan all slides up to the maximum magnification of 40x. These scanned images were then uploaded to an internet-based network (Slidepath). Digital images were captured for each sample at a magnification of
10x and stored. For both cohorts, once the images were captured, the red color of the stain was isolated using the eyedropper tool in Adobe Photoshop in each picture, and the background was faded to achieve contrast. Identical processing parameters were used for all sections in each experiment, regardless of treatment group. The entire image was then desaturated in order to convert it to black and white, imported to Image J, and converted to a binary image (Figure 11). The contrast analysis was performed on these binary images using the Analyze Particles command in Image J, which measured the proportion of area stained, number of separate plaques, and average plaque size for each image. For each animal, the values obtained for all images were averaged and the means for area stained, number of plaques, and average plaque size were used for further statistical analysis.
Figure 11. Preparing images for contrast analysis. The original image of hippocampus is shown photographed at 10x (A). First, the color of the plaques was selected and then copied and pasted as a second layer. The background layer was then converted to grayscale (B) and set to maximum lightness (C) in order to isolate the plaques. The entire image was then desaturated (D) and imported to ImageJ where it was converted into a binary image (E).

Statistical Analysis. To assess differences in plaque pathology between wild type and transgenic animals that received the mIL-1 Trap vs. the mFc, two-way ANOVAs (genotype x treatment) were conducted for each of the following measures: amyloid plaque proportion of area covered, plaque count, and average plaque size.
Results

**Plaque Burden Analysis.** The plaque burden contrast analysis revealed that swAPP/PS-1 transgenic mice had significantly more plaques (F(1,26)=13.49, p<.001), more total area covered by plaques (F(1,26)=210.06, p<.001), and a higher average plaque size (F(1,26)=58.12, p<.001) than the wild type animals. (Figure 13). Indeed, no Congo red-positive plaques were observed in any wild type animal (Figure 12). The contrast analysis did seem to pick up a small amount of background stain, which resulted in a small proportion of area covered by plaques to be reported as an artifact for wild type animals. Because the wild type animals did not show amyloid plaque pathology, they were excluded from subsequent plaque analyses. Three 2x2 ANOVAs were run with transgenic animals alone using treatment and cohort as factors. There was no significant difference between animals given mIL-1 trap versus mFc on the proportion of area covered by plaques (F(1, 14)=.102, p=754), number of plaques (F(1,14)=.002, p=.969), or average size per plaque (F(1,14)=.44, p=.516). There was a significant cohort effect for plaque area covered, plaque count, and average size per plaque such that cohort 2 had a greater area of the hippocampus covered by plaques, more plaques, and a larger average plaque size. However, there was no cohort by treatment interaction indicating that the treatment with mIL-1 Trap had no effect on either cohort. The differences between the cohorts on these measures may be due to a lighter overall stain with the second group and the different methods used to capture the images between the cohorts, both of which may have resulted in fewer plaques being detected in the contrast analysis for cohort 2.
Figure 12. Staining of representative hippocampal sections with Congo Red (photographed at 10x). Scale bar (blue) = 200µm
Figure 13. Results of contrast analysis for hippocampal plaque burden. Plaque burden was significantly increased for transgenic animals, with higher proportion of hippocampus covered by plaques (a), higher plaque counts (b), and bigger average plaque size (c). There were no differences between those treated with mFc vs. mIL-1 Trap on any of the plaque measures.
**Interim Discussion**

Based on prior literature, we initially hypothesized that chronic IL-1 inhibition would result in reduced amyloid plaque pathology. Past research has linked high levels of IL-1 to enhanced Aβ production and plaque deposition (Griffin et al., 1998, Shaftel et al., 2008). In fact, in one study, injection of synthetic IL-1β resulted in increased β-APP expression in rat brains (Sheng et al., 1996). However, our contrast analysis of amyloid plaques in the mouse hippocampus revealed no effect of the mIL-1 Trap. Long-term, systemic inhibition of IL-1β did not reduce overall hippocampal plaque burden and had no effect on number of plaques or their average size.

These plaque analysis results taken together with water maze data provide an interesting look at the nature of AD-like pathology in the swAPP/PS1 transgenic mice. The inhibition of IL-1β improved spatial learning in these mice without reducing the amount of amyloid plaques in the brain, suggesting that factors other than β-amyloid deposition are contributing to their cognitive deficits. One explanation is that the mIL-1 Trap may be improving memory by inhibiting the immune response to the plaques without reducing the plaques themselves. Therefore, the next goal of the study was to determine whether inhibition of IL-1 had any effect on components of the immune response that could explain the differences in performance on behavioral testing.
Chapter 5

MICROGLIA ANALYSIS

In addition to the main pathological hallmarks of AD, including amyloid plaques and hyperphosphorylated tau, the AD brain is characterized by a robust neuroinflammatory response that includes elevation of pro-inflammatory cytokines and activation of immune cells. In particular, microglia that express elevated levels of IL-1 are associated with amyloid plaques in brains of patients with AD (Griffin et al., 1989). Although the clustering of microglia around plaques suggests an attempt at phagocytosis, amyloid continues to build up as the disease progresses. Chronic elevations of IL-1 in the AD brain may cause microglia to remain in a sustained, classically activated state with potentially suboptimal clearance of Aβ and continued release of cytotoxic substances (Weldon et al., 1998).

In this study so far, transgenic mice treated with chronic systemic mIL-1 Trap demonstrated improved performance on the Morris Water Maze relative to mFc treated transgenic mice. However, the absence of any effect of the Trap on amyloid plaque pathology prompted us to look for effects on components of the immune that may explain the behavioral changes. Microglia are a prime target to explore because of the prominence of activated microglia, particularly those expressing IL-1, in the AD brain. Since IL-1β is known to trigger microglia to a classical activation state, long-term inhibition of IL-1β with the mIL-1 trap may reduce microglial activation or at least shift activation to a more phagocytic, anti-inflammatory M2 state. Because classical microglial activation is linked to impairments in learning and memory in PET studies (Edison et al., 2008; Yokokura et al., 2011), it is plausible that treatment with the mIL-1 Trap induced changes in microglial activation, which could potentially explain its
behavioral effects. In addition, M2 microglia are known to secrete elevated levels of growth factors, and these have the potential to induce beneficial neurotrophic effects.

One way to quantify the immune response using microglia would be to study them anatomically by looking at differences in the number of microglia as well as in microglial morphology. We expected that inhibition of IL-1 might result in fewer microglia present in the hippocampus, especially surrounding amyloid plaques. In addition, we had hypothesized that the animals treated with mIL-1 Trap would show alterations in the size of their microglia somas. In order to examine differential response to amyloid plaques, we measured size for microglia that were adjacent to plaques as well as those that were farther away. Differences between mFc-treated and Trap-treated animals on these measures would indicate that IL-1 inhibition altered the microglial profile in response to the plaques even if the plaques themselves were not reduced.

Methods

Iba-1 immunostaining. In order to assess characteristics of microglia in these mice, an Iba-1 immunostain was performed. Microglia express Iba-1 even at low levels of activation, which makes it an appropriate antibody for assessing microglial presence and morphology. Sections were immunostained in a 1:12 series using Iba-1 antibody (rabbit polyclonal, Millipore, Inc.) at a dilution of 1:500, with overnight incubation at 4° C. Sections were incubated with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) at a dilution of 1:1500 at room temperature for one hour. Avidin-biotin-peroxidase complex (ABC Elite Kit, Vector Laboratories) was applied for one hour at room temperature. The stain was then visualized using nickel-enhanced diaminobenzidine (DAB). To more precisely assess the relationship between
microglia and amyloid plaques, the Iba-1 immunostain was repeated with additional sections and counterstained with Congo Red in the second cohort.

**Microglia analyses.** The overall number of microglia in the hippocampus was estimated in both wild type and transgenic mice by first pseudorandomly sampling two fields of view in the CA1, CA3, and dentate gyrus each. An acetate sheet with a 45µm by 45µm grid of dots was placed over the screen at 10x and all microglial profiles within the plane of focus that fell on a dot were counted. The average number of microglial profiles per field was averaged for all of the fields and analyzed statistically.

The next step of the microglia analysis was to measure overall changes in microglia soma size to evaluate potential differences in microglial phenotype for those glia contacting amyloid plaques. As microglia become activated, their shape changes. The cell body enlarges and processes retract as the cell shifts from a resting state with surveillance properties to an activated cell ready for phagocytosis. Microglia activated to a classical or M1 phenotype express pro-inflammatory cytokines such as IL-1β as well as nitric oxide synthase (NOS) and other reactive nitrogen and oxygen species that can lead to neurotoxicity and damage to the extracellular matrix. On the other hand, microglia expressing an alternative or M2 phenotype are anti-inflammatory, release markers arginase-1, IL-10, and TGF-β, and are involved in phagocytosis (M2a phenotype), deactivation of glial inflammation, and wound healing (M2c phenotype; Varnum and Ikezu, 2012). Slides containing tissue double-stained with Iba-1 and Congo Red were scanned at high resolution. In each transgenic animal, images of plaques were captured within CA1, CA3, and the dentate gyrus of the hippocampus. In order to randomly select plaques to be imaged and measured, an acetate sheet with a 45µm x 45µm grid was laid over the screen with the tissue zoomed at 10x over a region of the hippocampus. Any plaque falling on
any dot in the grid was selected for measurement. These images were then taken at 40x and imported to Image J by an experimenter blind to the animal genotypes and treatments. This method yielded about 4-7 plaque images per area per animal.

For each image, the diameter and area of the plaque were measured. Only plaques with a diameter of 10µm or bigger were used. For each plaque, the soma of three proximities of microglia were traced and analyzed for soma area and sphericity. All visible microglia that were physically touching the plaque were considered plaque-adjacent. Microglia that were close to plaques, but not in physical contact with them, were considered plaque-proximal. To identify plaque-proximal microglia, a circle was drawn around the plaque in Image J 30µm from the plaque edge on all sides. All microglia that were within this circle, but not touching the plaque were considered plaque-proximal. In addition, microglia not associated with plaques (completely outside the circle but still within the field of view) were measured within each region for comparison to peri-plaque microglia in order to see whether the mIL-1 Trap differentially affected morphology of peri-plaque vs. plaque-distant microglia. Microglia were excluded from the plaque-distant measurements if they were within 30µm of another plaque or within 30µm of the edge of the image.

Microglia soma size was also measured in the hippocampus of the wild type animals in order to establish a baseline for comparison for the plaque-distant microglia. Although we would ideally obtain measurements of wild type mice treated with both mFc and mIL-1 Trap, technical problems with the scanner prevented us from obtaining some images from the double stained Iba-1/Congo Red slides. Therefore, microglia were measured in the wild type-mFc group to serve as a reference point.
For each plaque used in the microglia volume analysis, the number of adjacent microglia was also recorded and then combined for each animal in order to generate an average number of microglia per plaque. The same was done for the proximal microglia. These values were then used for statistical analysis to assess whether the number of microglia that were either near or touching plaques differed between transgenic animals treated with mIL-1 Trap vs. mFc. These measures could obviously not be conducted for wild type animals, because no wild type animal exhibited plaques.

**Hippocampal Volume.** Finally, in order to ensure that any differences in microglial density were not due to shrinkage of the tissue, measurements were taken for hippocampal volume. This measure also allowed us to evaluate any hippocampal atrophy that could have occurred as a consequence of either chronic IL-1 Trap treatment or of expression of the Alzheimer’s transgenes. Sections were stained with Cresyl Violet for detection of Nissl bodies. Hippocampi were measured using the Cavalieri Probe in StereoInvestigator (MBF Bioscience). Anterior hippocampus sections in a 1:6 series were selected and traced for each animal throughout the dorsal hippocampus. Six bilateral sections of hippocampus were measured for each animal. For each section, a grid pattern (of grid size 50 x 50µm²) was randomly laid over the tissue and all grid points falling within the traced area were selected. The probe estimated the volume of this section of hippocampus for each animal.

**Statistical analysis.** To compare microglial counts, the average number of microglial profiles per field in each hippocampal area (CA1, CA3, and dentate gyrus) were averaged to yield one number for each animal. A two-way ANOVA was then conducted using genotype and treatment as independent variables. For the microglial volume analysis, after the microglia somata were traced for each image, the values were combined for each animal for each category
(plaque-adjacent, proximal, and distant). The microglia volume values were then analyzed to detect any outliers, defined as greater than two standard deviations from the mean for each animal in each category. Any outliers were removed from further analysis. To assess differences in microglia soma area, a 2x3 factorial ANOVA was conducted using treatment and distance from plaque as independent variables. This analysis was conducted for whole hippocampus.

The number of microglia that were adjacent to and proximal to each plaque used in the volume measurement was averaged for each animal. A t-test was conducted for each of these values to compare the mFc group and the mIL-1 group.

Finally, the hippocampal volume estimates obtained from the Cavalieri probe were analyzed using a two-way ANOVA with genotype and treatment as independent variables.

Results

**Microglia density and distribution.** There was a slight statistical trend toward transgenic animals having more microglia in the hippocampus overall (F=3.263, p=.094). However, there was no effect of treatment (F=2.666, p=.126) and no genotype by treatment interaction (F=.171, p=.686) (Figure 15a). We ran additional analyses to determine whether there were differences in microglial density between groups for three hippocampal sub-regions. A similar pattern was found in CA3 with transgenic animals showing a trend towards a higher number of microglia than wild type animals (F=3.690, p=.077) but no effect of mIL-1 Trap treatment (F=1.794, p=.203) and no interaction (F=.000, p=.986). In CA1 and the DG, there was no difference between genotypes (CA1: F=2.021, p=.179; DG: F=1.758, p=.208), no difference
between treatment groups (CA1: F=1.290, p=.277; DG: F=2.225, p=.160) and no interactions (CA1: .368, p=.554; DG: F=.123, p=.731).

Although the overall number of hippocampal microglia present did not differ between groups, observation of the hippocampus of wild type vs. transgenic animals revealed a difference in distribution of the cells. Whereas wild type animals showed a uniform distribution of microglia, transgenic animals showed clusters of microglia throughout the hippocampus (Figure 14 a-b). A second Iba-1 immunostain counterstained with Congo Red confirmed that these microglia were clustered around amyloid plaques (Figure 14c). To assess whether the treatment with IL-1 Trap affected this recruitment of microglia to the amyloid plaques, the average number of microglia adjacent to (soma touching) plaques in the hippocampus was compared for transgenic animals across treatment groups. There was no difference between transgenic animals treated with mFc and mIL-1 Trap in average number of plaque-adjacent microglia (soma touching the plaque, t(6)=−1.045, p=.336) or plaque-proximal (microglia within 30µm of a plaque but not touching it, t(6)=.940, p=.383) (Figure 15 b-c).

We then examined whether number of microglia adjacent to and proximal to plaques differed between treatment groups in the CA1, CA3, and DG regions of the hippocampus. There was a statistical trend toward fewer plaque-proximal microglia in CA3 for the mIL-1 Trap-treated animals compared to mFc-treated animals (t(6)=2.249, p=.066). There were no other differences in number of plaque-adjacent or plaque-proximal microglia between the treatment groups in any of these regions.
Figure 14. Representative images of staining of hippocampus with Iba-1 and Congo Red. 
a-b) Images of hippocampus stained for Iba-1 photographed at 10x. a) Wild type animal with evenly distributed microglia. b) Transgenic animal showing clusters of microglia. c) Hippocampus of a transgenic animal immunostained with Iba-1 for microglia and counterstained with Congo Red for amyloid shows microglia clustering around amyloid plaques. Scale bar (yellow) = 200µm
Figure 15. Number of microglia per field and per plaque in the hippocampus

a) The overall estimates of hippocampal microglia showed a trend towards transgenic animals having more microglia present (p=.094), but there was no effect of treatment. b) The number of plaque-adjacent (touching) microglia per plaque did not differ by treatment. c) The number of plaque-proximal (within 30µm) microglia was also not affected by treatment.

Microglia soma size. The size of the somata were then measured and compared across treatment groups for cells adjacent, proximal, or distant to plaques. This comparison of microglia soma sizes revealed a significant effect of distance. Microglia that were plaque adjacent were bigger overall than proximal microglia, which were bigger than distant microglia (F(2,12)=62.592, p<.001). This finding implies that microglia adjacent to amyloid plaques were
in an activated, potentially phagocytic, state relative to plaque-distant microglia. When comparing transgenic animals treated with mFc vs. mIL-1 Trap, there was no overall difference in microglia size by treatment group (F(2,12)=.29, p=.609). However, there was an interaction between treatment and proximity (F(2,12)=16.13, p<.001). Animals treated with mIL-1 Trap had larger plaque-adjacent microglia than those of mFc treated animals. In contrast, the distant microglia were smaller for the mIL-1 Trap animals (Figure 17). For each transgenic animal, the average difference in size between plaque-adjacent and plaque-distant microglia was calculated and used for comparison between animals treated with mFc and mIL-1 Trap. Although all animals had positive difference scores, indicating larger plaque-adjacent relative to plaque-distant microglia, animals treated with the mIL-1 Trap showed significantly bigger differences in microglial size than animals treated with mFc (t(6)=4.865, p=.003) (Figure 16).

Figure 16. Representative staining of microglia with Iba-1 (purple) around amyloid plaques counterstained with Congo Red (red). Scale bar (yellow) = 50µm.
Figure 17. Average area of microglia by distance to amyloid plaques for each treatment group. Overall, there was a significant main effect of proximity with plaque-adjacent microglia having the largest somas and plaque-distant microglia having the smallest. There was a significant treatment by proximity interaction such that mIL-1 Trap animals had larger plaque-adjacent soma but smaller plaque-distant soma relative to mFc animals. Symbols: main effect of proximity ***p<.001, interaction shown as ### p<.001).

In order to provide a frame of reference, average microglia soma size was obtained for wild type mice treated with mFc. We found that transgenic animals in both treatment groups had larger plaque-distant microglia than the reference group. Therefore, although treatment with the mIL-1 Trap differentially affected microglial morphology in the hippocampus for the transgenic animals, hippocampal microglia in these animals were still more activated than in wild type animals. (Figure 18)
Figure 18. Comparison of plaque-distant microglia in transgenic mice to wild type mice treated with mFc. Plaque-distant microglia in transgenic mice of both treatment groups are larger than hippocampal microglia in wild type mice.

Additional analyses were conducted in order to evaluate microglia soma size in three areas of the hippocampus (CA1, CA3, and DG). Microglia in all three hippocampal regions showed the same effect of proximity to plaques with plaque-adjacent microglia having the largest somas and plaque-distant having the smallest (CA1: F(1,12)=36.089, p<.001; CA3: F(1,12)=21.711, p<.001; DG: F(1,12)=12.572, p=.001). Regarding the interaction between treatment and proximity, all three regions appear to show a similar pattern. However, this interaction is only significant in CA1 (F(1,12)=12.720, p=.001), shows a trend toward statistical significance in the DG (F(1,12)=3.556, p=.061) and is non-significant in CA3 (F(1,12)=.951, p=.414). There was no significant main effect of treatment on soma size in any of these hippocampal areas.

**Hippocampal volume.** Hippocampal volume was also measured using the Cavalieri probe. Results of the hippocampal volume analysis did not reveal any differences between transgenic and wild type animals (F(1,13)=.236, p=.635). Treatment with mIL-1 Trap did not
affect hippocampal volume (F(1,13)=.800, p=.387) and there was no interaction between

genotype and treatment (F(1,13)=.015, p=.903) (Figure 19).

![Estimated Hippocampal Volume](image)

**Figure 19.** Estimated hippocampal volume. Estimated hippocampal volume did not differ between groups

**Interim Discussion**

Although we found a trend towards more microglia in the hippocampus for transgenic animals, possibly reflecting greater immune activation associated with the disease state, microglial density was not affected by chronic IL-1 inhibition with the mIL-1 Trap. These results are consistent with a recent study that found that i.c.v. administration of IL-1ra did not affect microglial density in the dentate gyrus (Ben Menchem-Zidon, Ben Menahem, Ben Hur, & Yirmiya, 2014). However, that study did demonstrate a significant reduction of the number of microglia associated with or touching amyloid plaques in IL-1ra treated mice. We expected that administration of the mIL-1 Trap might also significantly reduce the number of plaque-adjacent microglia in the hippocampus but this was not the case. Despite the lack of effect of IL-1
inhibition on number of microglia, there were differences seen in the sizes of the somata between the treatment groups. Specifically, mIL-1 Trap-treated animals had larger plaque-adjacent microglia than mFc-treated animals, but smaller plaque distant microglia.

It might seem counterintuitive that microglia that were adjacent to plaques were actually bigger in trap treated animals than mFc treated animals. Since the somata enlarge as microglia go from a quiescent or ramified state to a more activated state, and IL-1 is known to activate microglia, one might assume that inhibiting IL-1 would lead to smaller somata. Perhaps the key finding is the difference in size between microglia that were physically touching plaques and microglia that are distant from plaques. Although there was an overall effect of plaque distance on soma size across all transgenic animals, indicating that microglia recruited to the site of the plaques were in a more activated state, mIL-1Trap-treated animals showed a significantly greater size difference between these plaque-adjacent and plaque-distant microglia compared to mFc-treated animals. Perhaps treatment with mIL-1 Trap resulted in microglia that were less active overall but that more efficiently responded to the amyloid plaques. In contrast, the microglia of mice treated with mFc were more reactive in general, but not as responsive to plaques.

The question remains of whether these differences in microglia size are related to behavioral performances and, if so, by what underlying mechanism. The larger size of the plaque-adjacent microglia in Trap-treated animals in this study might indicate a shift to an M2 activation state, which is generally anti-inflammatory, pro-neurotrophic, and phagocytic (Medeiros et al., 2013). However, if the larger microglia represent a more phagocytic phenotype, one would expect the number of amyloid plaques in the brain to decrease as the microglia clear the amyloid from the brain. As we previously showed, the plaque burden was unaffected by treatment with mIL-1 trap, despite the differences in microglia. It is possible that
microglia in mIL-1 Trap-treated animals are clearing plaques faster, but are also depositing amyloid faster due to other effects of the drug, resulting in no net difference in plaque burden. Depending on the study, IL-1 signaling has been shown to either interfere with microglia-mediated plaque clearance (Heneka et al., 2010) or facilitate it (Shaftel et al., 2007b), but reductions in plaque burden on its own has not consistently been accompanied by cognitive improvements and vice versa. In fact, a study by Kitazawa et al. (2011) that found that inhibiting IL-1 signaling in 3xTG-AD mice improved water maze performance and led to an increase in phagocytic activation, as measured by YM-1 and arginase-1, but had only a minimal effect on amyloid plaque burden. The results of the present study are consistent with these past findings and suggest that improvements in learning and memory performance are not directly caused by phagocytosis and clearance of amyloid plaques.

Perhaps the activated microglia in the Trap-treated animals are unable to clear the amyloid more efficiently, but are still beneficial due to other effects of an M2 phenotype. For example, a recent study showed that administration of IL-1ra to transgenic mice increased cell expression of BDNF and rescued hippocampal neurogenesis impairments, along with its beneficial effects on memory performance (Ben Menachem-Zidon et al., 2014). It may be that the IL-1 Trap in this study is improving cognition by impacting neurons, via secretion of growth factors like BDNF or promotion of neurogenesis. In addition, the Trap could be disrupting the pro-inflammatory IL-1-mediated cascade, inhibiting other pro-inflammatory cytokines and sparking release of anti-inflammatory substances, which could lead to a variety of downstream effects. More research will be needed with the mIL-1 trap in order to fully answer these questions, including staining for markers of M1 vs. M2 activation, BDNF, markers of neurogenesis, and other pro- or anti-inflammatory substances.
Chapter 6

CORRELATIONS BETWEEN BEHAVIOR AND ANATOMY

The final step of the study was to analyze the relationship between the behavioral and anatomical measures in these animals to determine whether changes in amyloid plaque pathology or microglial characteristics were predictive of changes in ability to learn.

Methods

A regression analysis was performed using quantitative measures of amyloid plaque burden (total plaque area, plaque number, plaque size) and measures of learning (water maze acquisition average latency) and memory (water maze retention proportion of time in goal quadrant and platform crossings). In addition, measures of microglial number and size were also correlated with these water maze measures, but only for the second cohort due to the unavailability of microglial values from cohort 1. The analyses were conducted first with all transgenic animals. The analyses were then conducted separately for transgenic animals treated with mFc and for animals treated with mIL-1 Trap to determine if the correlations differed between the groups, suggesting a dissociation of the relationships when IL-1 is inhibited.

Because there was a significant difference in overall plaque burden in transgenic animals from cohort 1 to cohort 2 (with cohort 1 showing more plaque pathology), but no interaction of cohort with treatment, a z transformation was conducted for the plaque burden values in order to better assess the relationships between amyloid burden and other measures for the two cohorts combined. Values for total plaque proportion, plaque number, and plaque size for each animal
were converted into z scores using the means and standard deviations for each animal’s treatment group and cohort. These z scores were then used to correlate with other measures.

**Results**

**Plaque burden vs. water maze measurements.** The correlation analyses revealed a significant negative relationship between average latency on water maze acquisition and the number of platform crosses on water maze retention ($r(16)=-.494$, $p=.037$), such that animals that performed worse on the acquisition phase (took longer to find the platform) tended to perform worse on the retention portion (made fewer platform crosses). In contrast, the average latency to find the platform did not correlate significantly with proportion of time spent in the goal quadrant during retention. In addition, the proportion of plaques in the hippocampus correlated significantly with plaque count ($r(16)=.819$, $p<.001$), such that having a higher number of plaques was related to a higher overall proportion of the hippocampus covered by plaques. There were no significant correlations between any of the plaque measurements (proportion, count, or average size) and any of the cognitive measures on water maze acquisition or retention (see Table 2).
Table 2. Correlations between plaque burden and water maze measurements for all transgenic animals

<table>
<thead>
<tr>
<th></th>
<th>WM Aquis (average latency)</th>
<th>WM Retent (prop in goal)</th>
<th>WM Retent (platform crosses)</th>
<th>Plaque Proportion z</th>
<th>Plaque Count z</th>
<th>Plaque size z</th>
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<tr>
<td>WM latency</td>
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<td>--</td>
<td>-.262</td>
<td>-.324</td>
<td>.121</td>
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<tr>
<td>Plaque prop z</td>
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<td>-.334</td>
<td>-.262</td>
<td>--</td>
<td>.819**</td>
<td>-.033</td>
</tr>
<tr>
<td>Plaque count z</td>
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<td>-.049</td>
<td>-.324</td>
<td>.819**</td>
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<td>-.422</td>
<td>.121</td>
<td>-.033</td>
<td>-.416</td>
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Notes. *p<.05, **p<.01

Next, the correlations were performed separately for transgenic mice treated with mFc and transgenic mice treated with mIL-1 Trap (see tables 3 and 4). The mFc mice had a significant positive correlation between average latency on water maze acquisition and plaque proportion, (r(7)=.673, p=.047), such that higher overall plaque burden corresponded to longer latencies to escape the platform during the acquisition phase. However, when looking at only animals treated with mIL-1 Trap, there was no longer any significant correlation between water maze acquisition and plaque proportion (r(7)= .268, p=.485) (Figure 20).

Similar to the results for all transgenic animals combined, those treated with mFc showed a strong negative correlation between water maze acquisition and platform crosses on the retention portion with worse performance on acquisition correlating with fewer platform crosses on retention. Again, there was no significant relationship between acquisition and the proportion of time spent in the goal quadrant in retention. In contrast, there was no correlation between
water maze acquisition and either of the retention variables for mIL-1 Trap-treated animals alone.

The proportion of hippocampus covered by plaque was positively correlated with the number of plaques in both mFc (r(7)=.938, p<.001) and mIL-1 Trap (r(7)=.700, p=.036) animals. For mFc animals alone, plaque size and count were significantly negatively related (r(7)=-.735, p=.024), such that the more plaques were present, the smaller they were. However, no such relationship was found in the mIL-1 Trap animals (r(7)=-.097, p=.803).

Table 3: Correlations between plaque burden and water maze measurements for transgenic – mFC animals

<table>
<thead>
<tr>
<th></th>
<th>WM Avg Lat</th>
<th>WM prop goal</th>
<th>WM plat cross</th>
<th>Plaque Prop z</th>
<th>Plaque Count z</th>
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<td>WM prop ingoal quad</td>
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<td>--</td>
<td>.251</td>
<td>-.104</td>
<td>.111</td>
<td>-.430</td>
</tr>
<tr>
<td>WM plat. cross</td>
<td>-.874**</td>
<td>.251</td>
<td>--</td>
<td>-.443</td>
<td>-.214</td>
<td>-.314</td>
</tr>
<tr>
<td>Plaque prop. (z)</td>
<td>.673*</td>
<td>-.104</td>
<td>-.443</td>
<td>--</td>
<td>.938***</td>
<td>-.461</td>
</tr>
<tr>
<td>Plaque count (z)</td>
<td>.490</td>
<td>.111</td>
<td>-.214</td>
<td>.938***</td>
<td>--</td>
<td>-.735*</td>
</tr>
<tr>
<td>Plaque size (z)</td>
<td>.080</td>
<td>-.430</td>
<td>-.314</td>
<td>-.461</td>
<td>-.735*</td>
<td>--</td>
</tr>
</tbody>
</table>

Notes. *p<.05, **p<.01, ***p<.001
Table 4: Correlations between plaque burden and water maze measurements for transgenic-mIL-1 Trap animals

<table>
<thead>
<tr>
<th></th>
<th>WM Aquis (average latency)</th>
<th>WM Retent (prop in goal)</th>
<th>WM Retent (platform crosses)</th>
<th>Plaque Proportion z</th>
<th>Plaque Count z</th>
<th>Plaque size z</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM latency</td>
<td>--</td>
<td>-.291</td>
<td>-.281</td>
<td>.268</td>
<td>-.129</td>
<td>.326</td>
</tr>
<tr>
<td>WM – prop goal</td>
<td>-.291</td>
<td>--</td>
<td>.024</td>
<td>-.610</td>
<td>-.239</td>
<td>-.416</td>
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<tr>
<td>WM – plat cross</td>
<td>-.281</td>
<td>.024</td>
<td>--</td>
<td>-.135</td>
<td>-.412</td>
<td>.443</td>
</tr>
<tr>
<td>Plaque prop (z)</td>
<td>.268</td>
<td>-.610</td>
<td>-.135</td>
<td>--</td>
<td>.700*</td>
<td>.394</td>
</tr>
<tr>
<td>Plaque count (z)</td>
<td>-.129</td>
<td>-.239</td>
<td>-.412</td>
<td>.700*</td>
<td>--</td>
<td>-.097</td>
</tr>
<tr>
<td>Plaque size (z)</td>
<td>.326</td>
<td>-.416</td>
<td>.443</td>
<td>.394</td>
<td>-.097</td>
<td>--</td>
</tr>
</tbody>
</table>

Notes: *p<.05

Figure 20. Plaque proportion (z score) vs. average water maze latency for each treatment group. For transgenic animals treated with mFc, a greater proportion of the hippocampus covered by amyloid plaque was significantly correlated with longer latencies to escape to the platform in water maze acquisition. There was no significant relationship between the two variables for the transgenic animals treated with mIL-1 Trap.
Microglia vs. water maze measurements. A regression analysis was conducted including the three water maze measures as well as measures of microglia size and activation (average number of adjacent microglia per plaque, size of microglia adjacent to, proximal to, and distant from plaques, and the difference in size between the adjacent and distant microglia).

There was a significant positive correlation between size of plaque-proximal microglia and the number of platform crosses on water maze retention ($r(6) = .747$, $p = .033$). However, none of the other microglial measures correlated with any of the cognitive measures.

Table 5: Correlations between measures of microglia number and size and water maze measurements for all transgenic animals

<table>
<thead>
<tr>
<th></th>
<th>WM Avg Lat.</th>
<th>WM prop goal</th>
<th>WM Plat Cr</th>
<th>Micro # Adj</th>
<th>Micro # Prox</th>
<th>micro size Adj</th>
<th>Micro size Prox</th>
<th>Micro size Dist</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water Maze Avg Latency</td>
<td>--</td>
<td>-.194</td>
<td>-.494*</td>
<td>-.108</td>
<td>-.059</td>
<td>-.223</td>
<td>-.062</td>
<td>.240</td>
</tr>
<tr>
<td>WM Prop in goal Quad</td>
<td>-.194</td>
<td>--</td>
<td>.129</td>
<td>-.267</td>
<td>.108</td>
<td>-.266</td>
<td>-.095</td>
<td>-.043</td>
</tr>
<tr>
<td>WM plat. crosses</td>
<td>-.494*</td>
<td>.129</td>
<td>--</td>
<td>-.372</td>
<td>.402</td>
<td>-.324</td>
<td>.747*</td>
<td>.333</td>
</tr>
<tr>
<td>Microglia # plaq-adjacent</td>
<td>-.108</td>
<td>-.267</td>
<td>-.372</td>
<td>--</td>
<td>.464</td>
<td>.472</td>
<td>.228</td>
<td>.282</td>
</tr>
<tr>
<td>Microglia # prox</td>
<td>-.059</td>
<td>.108</td>
<td>.402</td>
<td>.464</td>
<td>--</td>
<td>-.119</td>
<td>.571</td>
<td>.603</td>
</tr>
<tr>
<td>Microglia Size adjacent</td>
<td>-.223</td>
<td>-.266</td>
<td>-.324</td>
<td>.472</td>
<td>-.119</td>
<td>--</td>
<td>-.038</td>
<td>-.606</td>
</tr>
<tr>
<td>Microglia Size proximal</td>
<td>-.062</td>
<td>-.095</td>
<td>.747*</td>
<td>.228</td>
<td>.571</td>
<td>-.038</td>
<td>--</td>
<td>.556</td>
</tr>
<tr>
<td>Microglia size distant</td>
<td>.240</td>
<td>-.043</td>
<td>.333</td>
<td>.282</td>
<td>.603</td>
<td>-.606</td>
<td>.556</td>
<td>--</td>
</tr>
</tbody>
</table>

Notes. *$p<.05$, **$p<.01$, ***$p<.001$

When looking at each treatment group separately, different patterns of relationship between learning & memory and microglial parameters emerged. For mFc animals alone, the
size of plaque-distant microglia was negatively correlated with water maze retention platform crosses ($r(2)=-.954$, $p=.046$), such that the larger the microglia, the fewer platform crosses they made. Not only did mIL-1 Trap treated animals not show the same pattern, the relationship between size of distant microglia and platform crosses was actually positive ($r=.891$), although it did not reach significance ($p=.109$) (Figure 21b). In addition, mIL-1 Trap treated animals showed a significant relationship between the average number of plaque-adjacent microglia and proportion of time spent in the goal quadrant during water maze retention ($r(2)=.955$, $p=.045$), indicating that more microglia around plaques corresponded to better performance on this aspect of water maze retention. mFc-treated animals seemed to show the opposite pattern ($r=-.847$), but the relationship was not significant ($p=.153$) (Figure 21a).

A look at the correlations between average soma sizes of microglia that were adjacent, proximal, and distant to plaques also revealed a difference between treatment groups. For mFc animals, the size of plaque-proximal microglia positively correlated with the size of plaque-adjacent microglia ($r(2)=.957$, $p=.043$), but not to plaque distant microglia ($r(2)=-.199$, $p=.801$). For mIL-1 Trap animals, though, the plaque-proximal microglia size correlated significantly to the plaque-distant size ($r(2)=.957$, $p=.043$), but not to plaque-adjacent microglia ($r(2)=.099$, $p=.901$). In other words, the size of microglia that were near plaques was more similar to plaque-adjacent microglia for mFc animals, but more similar to plaque-distant microglia for mIL-1 Trap animals. This finding may support the idea that microglial activation was more targeted to plaques in mIL-1 Trap animals but more generally reactive in mFc animals.
Table 6. Correlations between measures of microglia number and size and water maze measurements for transgenic animals treated with mFc

<table>
<thead>
<tr>
<th></th>
<th>WM Avg Lat.</th>
<th>WM prop goal</th>
<th>WM Plat Cr</th>
<th>Micro # Adj</th>
<th>Micro# Prox</th>
<th>micro size Adj</th>
<th>Micro size Prox</th>
<th>Micro size Dist</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water Maze Avg Latency</td>
<td>--</td>
<td>-.107</td>
<td>-.874**</td>
<td>.898</td>
<td>.368</td>
<td>.046</td>
<td>-.177</td>
<td>.619</td>
</tr>
<tr>
<td>WM Prop in goal Quad</td>
<td>-.107</td>
<td>--</td>
<td>.251</td>
<td>-.847</td>
<td>-.904</td>
<td>-.686</td>
<td>-.624</td>
<td>-.558</td>
</tr>
<tr>
<td>WM plat. crosses</td>
<td>-.878**</td>
<td>.251</td>
<td>--</td>
<td>-.831</td>
<td>-.076</td>
<td>.304</td>
<td>.370</td>
<td>-.954*</td>
</tr>
<tr>
<td>Microglia # adjacent</td>
<td>.898</td>
<td>-.847</td>
<td>-.831</td>
<td>--</td>
<td>.598</td>
<td>.256</td>
<td>.121</td>
<td>.792</td>
</tr>
<tr>
<td>Microglia # near</td>
<td>.368</td>
<td>-.904</td>
<td>-.076</td>
<td>.598</td>
<td>--</td>
<td>.927</td>
<td>.850</td>
<td>.150</td>
</tr>
<tr>
<td>Microglia Size adjacent</td>
<td>.046</td>
<td>-.686</td>
<td>.304</td>
<td>.256</td>
<td>.927</td>
<td>--</td>
<td>.957*</td>
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<tr>
<td>Microglia Size proximal</td>
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<td>-.199</td>
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<tr>
<td>Microglia size distant</td>
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<td>-.558</td>
<td>-.954*</td>
<td>.792</td>
<td>.150</td>
<td>-.210</td>
<td>-.199</td>
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</tr>
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</table>

Notes. *p<.05, **p<.01,
Table 7. Correlations between measures of microglia number and size and water maze measurements for transgenic animals treated with mIL-1 Trap

<table>
<thead>
<tr>
<th></th>
<th>WM Avg Lat.</th>
<th>WM prop goal</th>
<th>WM Plat Cr</th>
<th>Micro # Adj</th>
<th>Micro# Prox</th>
<th>micro size Adj</th>
<th>Micro size Prox</th>
<th>Micro size Dist</th>
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<tr>
<td>Water Maze Avg Latency</td>
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<td>-.281</td>
<td>-.679</td>
<td>-.308</td>
<td>.097</td>
<td>-.110</td>
<td>-.202</td>
</tr>
<tr>
<td>WM Prop in goal Quad</td>
<td>-.291</td>
<td>--</td>
<td>.024</td>
<td>.955*</td>
<td>.791</td>
<td>.467</td>
<td>.296</td>
<td>.505</td>
</tr>
<tr>
<td>WM plat. crosses</td>
<td>-.281</td>
<td>.024</td>
<td>--</td>
<td>.350</td>
<td>.440</td>
<td>.222</td>
<td>.938</td>
<td>.891</td>
</tr>
<tr>
<td>Microglia # adjacent</td>
<td>-.679</td>
<td>.955*</td>
<td>.350</td>
<td>--</td>
<td>.893</td>
<td>.545</td>
<td>.540</td>
<td>.731</td>
</tr>
<tr>
<td>Microglia # proximal</td>
<td>-.308</td>
<td>.791</td>
<td>.440</td>
<td>.893</td>
<td>--</td>
<td>.850</td>
<td>.478</td>
<td>.711</td>
</tr>
<tr>
<td>Microglia Size adjacent</td>
<td>.097</td>
<td>.467</td>
<td>.222</td>
<td>.545</td>
<td>.850</td>
<td>--</td>
<td>.099</td>
<td>.354</td>
</tr>
<tr>
<td>Microglia Size proximal</td>
<td>-.110</td>
<td>.296</td>
<td>.938</td>
<td>.540</td>
<td>.478</td>
<td>.099</td>
<td>--</td>
<td>.957*</td>
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<tr>
<td>Microglia size distant</td>
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<td>.505</td>
<td>.891</td>
<td>.731</td>
<td>.711</td>
<td>.354</td>
<td>--</td>
<td></td>
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Note. *p < .05,
Figure 21. Correlations of size/number of microglia vs. water maze retention for both treatment groups. A) mIL-1 Trap-treated animals showed a significant positive correlation between the average number of plaque-adjacent microglia and proportion of time spent in the goal quadrant in water maze retention. In contrast, mFc-treated animals showed a negative, though nonsignificant, relationship. B) For mFc-treated animals, the size of plaque-distant microglia was significantly negatively correlated with number of platform crosses. However, mIL-1 Trap-treated animals showed a positive relationship between these variables.
Interim Discussion

To evaluate the role of amyloid plaques in cognition, we looked at the relationship between plaque deposits and water maze performance for transgenic animals. Blessed et al. (1968) first reported a correlation between the number of senile plaques in the neocortex and cognitive performance in Alzheimer’s patients. Since then, positron emission tomography (PET) using Pittsburgh Compound-B (PIB) has allowed researchers to more closely examine the relationship between AD and amyloid plaques in vivo (Klunk et al., 2004), but studies looking at the relationship between Aβ load and cognitive function have generally found either a weak correlation (Villemange et al., 2011) or no correlation (Edison et al., 2008; Yokokura et al., 2011). In the present study, when looking only at transgenic animals treated with mFc, we did find a significant correlation between water maze acquisition and plaque burden, with higher proportion of plaques in the hippocampus correlating with poorer performance (longer latencies to find platform). However, transgenic animals treated with mIL-1 Trap showed no significant relationship between these variables. As demonstrated in chapter 4, treatment with mIL-1 Trap did not alter the overall plaque burden, but here we have showed that it eliminated the significant relationship between those plaques and learning performance. This dissociation between cognitive function and plaque burden supports the hypothesis that it is the immune response to the plaques, rather than the plaques themselves, that are causing the cognitive impairments.

In order to assess the relationship between immune activation and cognitive performance, we then correlated our various microglia measurements with the same water maze measures. Unfortunately, these correlations could only be conducted with the second cohort, as microglia data from the first cohort were unavailable. Nonetheless, there were some interesting patterns that emerged. For animals treated with the mIL-1 Trap, higher numbers of plaque-adjacent
microglia corresponded significantly to more time spent in the goal quadrant during water maze retention whereas the opposite pattern was seen in animals treated with mFc. Also, the mFc animals showed a significant negative relationship between size of plaque-distant microglia and number of platform crosses with the opposite pattern in mIL-1 Trap animals. Taken together, these correlations seem to show a pattern where the presence of microglia and the size of the somata relate to negative memory performance in mFc animals but improved memory performance in mIL-1 Trap animals. It is interesting that these correlations are seen with water maze retention measures, since performance on these measures did not differ between the treatment groups overall (see Chapter 3). It appears as though treatment with mIL-1 Trap may have shifted the relationship between microglial activation and memory, without improving performance overall.

In addition, although most of the correlations between the cognitive measures and microglia measures are not significant for either the mFc or mIL-1 Trap groups, likely due to very small sample sizes, the pattern of correlations appears to be generally different between the two groups. The animals treated with mIL-1 Trap show all positive (although mostly nonsignificant) correlations between measures of microglial size/number and measures of water maze retention. In contrast, animals treated with mFc had mostly negative correlations between the same variables. A similar pattern can be seen in correlations between water maze latency and microglial measures, such as number of plaque-adjacent microglia, with mIL-1 Trap animals tending to show negative correlations (e.g. more microglia associated with faster latencies), but mFc animals showing positive correlations. These patterns are interesting and may warrant further study, but none of those measures reach statistical significance and thus, no conclusions can be drawn at this point.
Another consideration that must be mentioned is the question of whether administering mFc alone has any behavioral effects on these animals. mFc was chosen as a control as it is a component of the mIL-1 Trap but does not contain the IL-1 inhibiting properties. It is assumed that it has no significant effects on biology or behavior, but in order to definitively answer that question, a true treatment-naïve control group would be necessary. The correlations shown with the mFc group alone could potentially reflect subtle effects of chronic mFc administration.

These findings suggest that, although measures of microglial density and the number of microglia around plaques are not affected by mIL-1 Trap treatment, the presence of microglia is more beneficial after IL-1 Trap treatment. This relationship could suggest that the mIL-1 Trap shifts the microglia to an alternative activation phenotype that is generally more beneficial for hippocampal function. It is, however, still not clear whether the relationship shift toward microglia being more beneficial is due to more effective phagocytosis of amyloid (unlikely since plaque burden is unaffected), due to other aspects such as secretion of BDNF, or due to some as-yet unexplained effect. More research will be necessary in order to determine the exact nature of the relationship between microglia and cognitive functioning.
Chapter 7

DISCUSSION

In this study, we used systemic administration of the mIL-1 Trap to inhibit interleukin-1 signaling for 5 months after the onset of disease in order to observe its effects on both behavior and Alzheimer’s-like brain pathology. Our initial hypothesis was that IL-1 inhibition would improve performance on measures of learning and memory while reducing plaque burden. We found that animals treated with the mIL-1 Trap performed significantly better on a spatial learning task (water maze acquisition) although there was no difference on retention. However, treatment with the Trap had no effect on amyloid plaque burden in the hippocampus. We then investigated its effect on the immune response by analyzing hippocampal microglia. There was no difference between treatment groups in either the overall number of microglia in the hippocampus or the number of microglia adjacent (touching) or proximal (within 30µm) to amyloid plaques. It did alter the sizes of microglia somata in the hippocampus such that Trap-treated animals had larger plaque-adjacent microglia but smaller distal microglia. Finally, we looked at the relationship between the anatomical measures and performance on behavioral testing. Treatment with the mIL-1 Trap eliminated the significant positive relationship between plaque burden and cognitive impairments seen in mFc-treated transgenic mice. None of the microglial measures (number or soma size) significantly correlated with the behavioral measures.

Our behavioral findings are consistent with previous research showing that inhibiting inflammation can have a positive effect on cognition in AD. Specifically, He et al. (2011) showed that transgenic AD rats treated with tetrandine had both reductions of hippocampal IL-1β and TNFa and improved spatial memory. A recent study directly targeted IL-1 inhibition in TG mice by using intra-hippocampal transplantation of neural precursor cells overexpressing IL-1ra
found a complete reversal of memory impairments (Ben Menachem-Zidon et al., 2014). There is even some support for peripheral IL-1 inhibition improving memory function. Kitazawa et al. (2011) demonstrated improved water maze performance in aged 3xTg-AD mice after six months of intraperitoneal injections with IL-1 receptor blocking antibody. Interestingly, only a small proportion of the peripherally administered anti-IL-1R mAb was detected in the brain, raising questions of how much of the effects were due to direct CNS action versus peripheral effects of IL-1 inhibition. The findings of our study add to the story of IL-1’s role in AD. Most previous studies inhibiting IL-1 in transgenic rodents have used IL-1ra, but ours is the first to use the mIL-1 Trap, a more potent blocker of IL-1 action. We also showed an effect using subcutaneous injections, rather than intraperitoneal or intracerebral administration.

In addition to the behavioral results, we found that IL-1 inhibition had no effect on plaque burden in the hippocampus. This finding was somewhat surprising because past studies have implicated IL-1 in APP expression and processing as well as amyloid plaque deposition (Griffin et al., 1995, Sheng et al., 2003, Goldgaber et al., 1989). However, other recent studies have showed no reduction in plaque pathology after systemic inhibition of IL-1 in aged transgenic mice (Kitazawa et al., 2011). Taken together with the water maze data, these results suggest that other factors are contributing to cognitive deficits seen in Alzheimer’s disease. Although plaques are the hallmark pathological signs of AD, the relationship between plaques and cognition is controversial, with more and more researchers concluding that amyloid is not the main culprit. For example, studies using PIB have not consistently shown any correlation between plaque burden and performance on cognitive testing. In our study, there was a strong significant correlation between amount of hippocampal area covered by plaques and performance on the acquisition phase of the Water Maze, such that animals with worse plaque
pathology performed worse on the maze. However, in animals treated with the mIL-1 Trap, this relationship was eliminated. Although the plaques were still present, they were no longer associated with the animals’ cognitive performance. These findings fit with the growing body of evidence against the amyloid cascade hypothesis, and suggest that there are factors downstream of the plaques themselves that are causing the memory impairments in AD.

Because the amyloid pathology had not changed with IL-1 Trap treatment in our studies, the next step was to examine the immune response to the plaques. We chose to look at microglia because of the prominence of IL-1β-expressing microglia in the AD brain, especially surrounding amyloid plaques (Griffin et al., 1989). It is thought that this clustering of microglia suggests attempts to phagocytose and clear the amyloid deposits. However, these microglia remain in a classically activated state with impaired phagocytosis and increased expression of pro-inflammatory cytokines as well as harmful reactive oxygen and nitrogen species (Hickman et al., 2008; Weldon et al., 1998). In the current study, microglia that were adjacent to amyloid plaques had significantly larger somata than microglia that were not near plaques, indicating activation of the plaque-associated cells. However, animals treated with mIL-1 Trap had larger plaque-adjacent microglia and smaller plaque-distal hippocampal microglia compared to mFc treated animals. This discrepancy may indicate that Trap-treated animals have more quiescent, resting microglia in general, but they are more reactive in contact with amyloid. These larger somata may also indicate a shift to an M2 or alternative activation state. The M2 phenotype is thought to be more phagocytic, suggesting that the microglia in these Trap-treated animals are better suited to clear the amyloid plaques. However, this is contradicted by the finding that amyloid plaque burden was not changed in animals treated with mIL-1 Trap.
Finally, some correlations were observed between the measures of microglial number and morphology and certain behavioral measures, which differed between mFc and mIL-1 Trap-treated animals. In animals treated with the mIL-1 Trap, having more plaque-adjacent microglia and larger plaque-distant microglia both corresponded to better performance on water maze retention, whereas for animals treated with mFc, more plaque-adjacent microglia and larger plaque-distant microglia were associated with poorer retention performance. These differences seem to support the idea that the mIL-1 Trap shifted the microglia into an alternative activation phenotype in which activation is beneficial, rather than detrimental. As mentioned above, the fact that plaque burden did not decrease suggests that phagocytosis is not the underlying beneficial mechanism of the microglia, but perhaps the microglia are affecting behavior in other ways, such as through secretion of growth factors like BDNF. Ultimately, the role of microglia in AD pathology is still not clear and warrants further study.

Limitations

Although the current study showed a potentially protective role of systemic mIL-1 Trap treatment in the swAPP/PS-1 double transgenic model of Alzheimer’s Disease, there were a number of limitations to the study. For instance, this study just looked at one time point in the course of the Alzheimer’s-like pathology. It is possible that the effects of the drug may differ at different stages of the disease. In particular, starting the treatment course earlier might have been more effective in preventing the onset of AD-like symptoms. We chose 8 months as the starting point for treatment because this is when swAPP/PS1 first consistently show pathological signs of AD (Hickman et al., 2008) and water maze impairments (Reiserer et al., 2007). In humans, we can rarely predict the onset of AD before symptoms appear, and therefore we chose
Another potential limitation of our study was our choice to use systemic, rather than intracerebral, injections of mIL-1 Trap. This choice might have limited the effectiveness of the drug, given that the blood brain barrier could have prevented the drug from having its maximum effect. Although we did see an effect even with these systemic injections, we do not know if the drug is having its effects peripherally or centrally. If the drug had no measurable effects whatsoever, this question would become very important. We would need to determine whether the drug truly is truly not effective in this disease or whether the peripheral administration prevented it from reaching its target. Because there were significant effects found both in behavior and pathology, we have shown that the mIL-1 trap can be therapeutic even when given peripherally. Again, we chose this method of drug administration in order to increase clinical relevance. If the drug can be effective when injected subcutaneously, it would prove more promising as a human treatment because of the potential to avoid brain surgery for device implantation.

Another issue is that small sample sizes and high variability in this study may have interfered with our ability to detect significant results in some of our measures, particularly our microglia analyses which could only be conducted with one cohort because of failure of the tissue to immunostain microglia well in the first cohort. We might have increased the effect size, thereby partially compensating for the small sample size, if we had used higher or more frequent doses of the mIL-1 Trap. Previous studies using the same reagent in a mouse model of gout showed optimal efficacy at 35mg/kg three times a week (Torres et al., 2009). We were, however, hesitant to use these more aggressive dosing parameters in a 5-month treatment.
regimen in older mice given the potential for chronic mild immunosuppression. Future studies could, perhaps, probe the impact of higher or more frequent doses, especially given that our animals appeared grossly healthy.

Finally, the difference in background lineage for the experimental animals between the first and second cohort is a limitation. These differences in strain add overall variability to the study and could also account for some of the differences between cohorts. However the effect of treatment did not interact with any other variable for any measure such that the effects of the mIL-1 Trap were the same regardless of the background strain. Particularly, the fact that treatment with mIL-1 Trap had a similar effect on water maze performance for both cohorts is encouraging. Ideally the results should be repeated with mice from the same lineage to more fully elucidate the effects of IL-1 inhibition on behavior without the potential complication of background strain.

Future Directions

The findings presented here raise several questions and suggest directions for future research. One potential avenue for investigation is to more fully explore the effects of IL-1 inhibition on the immune response. The present study used an Iba-1 stain to look at microglia density and basic morphology. However, future studies should differentiate between classic (M1) and alternative (M2) microglia activation profiles. It is possible that the change in soma size of plaque-adjacent microglia in animals treated with mIL-1 Trap compared to mFc represents different activation phenotypes, but more targeted immunostaining with M1 associated markers such as iNOS, MHCII or TNFa and M2-associated markers like arginase or IL-10 would be needed to answer that question (Varnum and Ikezu, 2012).
The distinction between resident microglia and infiltrating peripheral macrophages has also been addressed in recent research studies. For example, Stalder et al. (2005) used green fluorescent protein to demonstrate that peripheral bone marrow-derived macrophages invade the CNS and surround amyloid plaques in transgenic APP23 mice. Since macrophages take on different activation phenotypes similar to what is seen in microglia, some have hypothesized that these invading peripheral cells are driving the difference between classical and alternative activation in the CNS (Ransohoff and Brown, 2012). Perhaps future studies with the mIL-1 Trap in AD could use similar GFP-labeling methods to differentiate between resident and peripheral cells.

In addition to immune cells, future studies should investigate the effects of the mIL-1 Trap on other markers of plasticity. A recent study demonstrated that impaired hippocampal neurogenesis in transgenic AD animals can be completely rescued by implantation with neural precursor cells expressing Il-1ra and partially reversed with intracerebroventricular IL-1ra administration (Ben Menachem-Zidon et al., 2014). This study also demonstrated a significant increase in endogenous hippocampal cells expressing the growth factor BDNF. Since IL-1 suppresses BDNF expression in the hippocampus (Barrientos et al., 2004), inhibiting IL-1 with the mIL-1 Trap might have increased hippocampal BDNF levels therefore promoting functional recovery. Finally, it may be important to examine the effect of IL-1 inhibition on synapses in the hippocampus and cerebral cortex. Synapse loss, as measured by reduced synaptophysin immunoreactivity, is seen in AD brains but has been shown to correlate with markers of inflammation, rather than with Aβ load (Lue et al., 1996). In fact, these markers of synapse loss and inflammation differentiated between AD patients and high pathology controls who exhibited amyloid plaques and neurofibrillary tangles but did not show overt dementia.
Another potential goal for future research is to determine the exact pathways by which peripheral inhibition of IL-1 can affect brain pathology and behavioral changes in AD animals. One possibility is that the trap is crossing over the blood brain barrier and directly inhibiting IL-1 expression in the brain. This explanation is unlikely since the Trap is a relatively large biological protein and can probably only cross the BBB in very small amounts. One way to address that question could be to stain for mFc in the brain, which should be relatively low in normal animals. Alternatively, one could measure IL-1 levels or mIL-1 Trap levels in the brain using assays, which could not be conducted in perfusion fixed tissue. Even if the Trap itself does not enter the brain, it could affect CNS indirectly through the peripheral nervous system. There are a number of pathways by which peripheral expression of IL-1 can induce expression in the brain, which explain how peripheral infections can induce sickness-related behaviors and altered cognition. Through the neural pathway, peripheral pathogens and cytokines stimulate primary afferent nerves which project to the nucleus tractus solitarius and then to structures of the hypothalamus, extended amygdala, and the periaqueductal grey (Dantzer et al., 2008).

Alternatively, circulating PAMPs can activate the humoral pathway through the choroid plexus and circumventricular organs, inducing production of pro-inflammatory cytokines, which then enter the brain by volume diffusion (Dantzer et al., 2008). Systemic administration of mIL-1 Trap could be impacting brain expression of IL-1 through one of these pathways. Finally, as mentioned before, IL-1 inhibition in the periphery could be altering activation of peripheral monocytes and macrophages affecting their recruitment to the CNS in response to plaque deposition. Further studies would need to be conducted in order to determine the exact method by which the mIL-1 Trap is impacting brain pathology and ultimately behavior.
Another way to explore the role of cytokines in AD is to use genetic methods of blocking IL-1, rather than pharmacological interventions. Our lab previously attempted to cross swAPP-PS1 mice with IL-1R1 knockout mice, which lack the main endogenous interleukin-1 receptor, in order to evaluate how a lack of interleukin signaling affected the disease progression. Although our attempts at breeding these two strains resulted in poor viability in the offspring, this could be a potential avenue for future research.

Finally, it would be interesting to track learning and memory over time in these animals using repeated behavioral testing. This sort of study could establish a timeline of cognitive decline and would be useful for targeting treatment to the point when cognitive impairments first appear. In addition, other measures of memory should be used in order to tease out why animals showed improved learning on water maze acquisition, but not retention. Perhaps using other hippocampal tasks in addition could help determine whether this water maze pattern reflects different strategies used by the mice or whether there is truly no effect on memory retention.

**Impact**

This study has potential implications for the understanding and treatment of Alzheimer’s Disease. First, the current study provides support for the growing hypothesis that amyloid plaques do not, themselves, cause the hallmark cognitive impairments of Alzheimer’s Disease. Indeed, it supports the idea that components of neuroinflammation, perhaps triggered in part by the presence of the plaques, are major pathogenic contributors. Finally, our data provide initial evidence that the IL-1 Trap could be a promising treatment for AD related cognitive impairments. The findings suggest that even large biological inhibitors of IL-1, given after
disease onset, could provide significant benefit to patients suffering from the profound cognitive impairments characterizing this devastating disease.
Chapter 8

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