Region-specific Distribution of $\beta$-Amyloid and Cytokine Expression in TgCRND8 Mice

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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Abstract

Alzheimer’s disease (AD) is a multifactorial disease that results in progressive neurodegeneration. Brain regions are differentially affected in AD; some are more vulnerable to degeneration than others. There is an age-dependent effect on beta-amyloid (Aβ) accumulation and neuroinflammation as disease progresses. In the TgCRND8 APP transgenic mouse model, levels of various Aβ species and cytokines were determined as a function of brain region and age. Aβ was found to accumulate in the brain prior to the sequential elevation of IL-1β and CXCL1. Levels of Aβ, IL-1β and CXCL1 were elevated in regions that are severely affected in AD patients. It has been shown for the first time in an APP transgenic model that CXCL1 elevation occurs following IL-1β elevation.
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List of Abbreviations

Alzheimer’s disease (AD)
Amyloid precursor protein (APP)
Amyloispheroid (ASPD)
APP intracellular domain (ACID)
Aβ-derived diffusible ligand (ADDL)
Beta-amyloid (Aβ)
Beta-secretase 1 (BACE1)
Cholinesterase inhibitor (ChEI)
Extracullular receptor kinase (ERK)
Glial fibrillary acid protein (GFAP)
Interferon gamma (IFN-γ)
Interleukin -1 beta (IL-1β)
Interleukin-1 receptor 1 (IL-1R1)
Long-term potentiation (LTP)
Phosphate buffered saline (PBS)
Phosphoinositide -3 kinase (PI-3K)
Toll-like receptor (TLR)
Tumor necrosis factor alpha (TNF-α)
Tumor necrosis factor receptor 1 (TNFR1)
Beta-amyloid (Aβ) accumulation and neuroinflammation are two characteristics of Alzheimer’s disease (AD). Aβ is over-expressed in AD and it aggregates to cause neurotoxicity (Lambert et al., 1998; Lesne et al., 2006; Noguchi et al., 2009). Aggregates of Aβ trigger the activation of microglia and astrocytes in the brain to cause neuroinflammation (Hu et al., 1998; Jimenez et al., 2008; McGeer et al., 1987; Qin et al., 2001). Inflammatory mediators such as IL-1, TNF-α and IFN-γ can in turn promote increased production of Aβ (Goldgaber, et al., 1989; Lahiri, et al., 1995; Liao et al., 2004; Yang, et al., 1998). In AD, some brain regions are more affected than others and Aβ accumulation is not uniform across all regions. This thesis examines Aβ accumulation in various brain regions as Aβ pathology develops in TgCRND8 mice, an APP transgenic mouse model, and associated regional neuroinflammation. Results from this thesis present a temporal pattern of Aβ accumulation, followed by elevations of inflammatory mediators IL-1β then CXCL1 in regions of high Aβ accumulation.

1.1 Alzheimer’s Disease

AD is a progressive neurodegenerative disorder of the central nervous system. It is the fourth leading cause of death in developed countries and it is also the most common cause of dementia in the elderly. There are many genetic mutations and polymorphisms associated with AD (Blacker et al., 2003). Early-onset or familial AD with genetic etiology develops before the age
of 65 and late-onset, usually sporadic AD, develops after the age of 65 (Bertram and Tanzi, 2005).

Alzheimer’s disease is a multifactorial disease. Pathologically, AD is characterized by extracellular deposition of beta-amyloid peptide (Aβ), intracellular neurofibrillary tangles due to hyperphosphorylation of tau and multiple neurotransmitter alterations (Selkoe, 1991). Aβ gradually deposits in the AD brain in five stages (Thal et al., 2002). In phase 1, Aβ first deposits in the neocortex. In phase 2, Aβ deposition extends to the CA1 region of the hippocampus and the entorhinal region. Affected regions in phase 3 consist of the dentate gyrus of the hippocampus, amygdala, thalamus, striatum, hylothalamus and the basal forebrain. In phase 4, CA4 of the hippocampus and the substantia nigra are affected. Aβ deposits last in the cerebellum at phase 5 (Thal et al., 2002). The olfactory system has also been reported as one of the earliest regions affected in AD in humans (Christen-Zaech et al., 2003). Brain regions that project to or from areas of high density Aβ plaques suffer the most neuronal and synaptic degeneration (reviewed in Kar and Quirion, 2004). Several types of Aβ plaques are found in the Alzheimer brain. Neuritic plaques contain a dense amyloid core as well as dystrophic neurites. Compact plaques, also known as “burnt-out” plaques consist of dense amyloid aggregates. Diffuse plaques consist of less compact amyloid deposits that are usually large and poorly circumscribed (Dickson, 1997).

Neuroinflammation is another characteristic of AD. Microglia, immune cells of the brain, are activated AD cases as imaged in vivo in mild to moderate AD patients (Cagnin et al., 2001). Immunohistochemical analyses of AD brains at autopsy show that microglia typically surround compact Aβ plaques (Sasaki et al., 1997). Astrocytes, also capable of secreting inflammatory
cytokines, are activated in the AD brain and they are found most often in neuritic plaques (Mrak et al. 1996). Glial activation causes the release of reactive oxygen species, cytokines and chemokines and these inflammatory mediators are thought to cause neuronal damage (Mattson et al., 1997). However, cytokines involved in AD such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and interleukin 1-β (IL-1β) have also shown to have neuroprotective effects (Mattson et al., 1997; Shaftel et al., 2007; Nakashima et al., 1999).

The most commonly used drugs in treatment of Alzheimer’s disease are cholinesterase inhibitors (ChEIs). As cholinergic neurons are damaged and acetylcholine levels are reduced in AD, ChEIs compensate for the loss of cholinergic innervation by inhibiting the degradation of acetylcholine thus increasing the amount of acetylcholine in the synapses. ChEIs include rivastigmine (Exelon, Novartis), donepezil (Aricept, Pfizer) and galantamine (Reminyl, Janssen Pharmaceutica). In addition to acting as a ChEI, rivastigmine has also been shown to reduce neuroinflammation in experimental autoimmune encephalomyelitis by reducing microglial activation and levels of TNF-α, INF-γ and IL-17 (Nizri et al., 2008). Rivastigmine could also be beneficial for treating AD because it has been shown to increase mitochondrial oxidative phosphorylation to ameliorate the dysfunctional mitochondrial electron transport chain in AD (Casademont et al., 2003).

Another strategy used to treat Alzheimer’s disease is to reduce neuroinflammation. Ibuprofen has shown some beneficial effects as an anti-inflammatory agent. Non-steroidal anti-inflammatory drugs were extensively studied but prolonged use leads to several side effects (Lahiri et al., 2003). PPARγ agonists have also been used to reduce the neuroinflammation
associated with AD but effective penetration through the blood-brain barrier has been a challenge (Maeshiba et al., 1997).

1.2 Beta-amyloid

1.2.1 APP Processing

Amyloid precursor protein (APP) is a membrane-spanning polypeptide that is processed through proteolysis into many peptide fragments, one of which is Aβ. Figure 1 illustrates the APP cleavage process. In the non-amyloidogenic pathway, APP is first cleaved by α-secretase followed by the γ-secretase complex, which produces a p3 peptide from the C-terminal fragment of 83 amino acids long (C83). Since α-secretase cleavage occurs between amino acid residues 16 (Lys) and 17 (Leu) of the Aβ peptide sequence, no Aβ is secreted via the α-secretase non-amyloidogenic pathway (Esch et al., 1990). On the other hand, APP cleavage via β-secretase followed by the γ-secretase complex is amyloidogenic, producing Aβ from the C-terminal fragment of 99 amino acids (C99) (reviewed by Sisodia and St. George-Hyslop, 2002). Since C83 and C99 cleaved by the γ-secretase complex respectively produce the p3 fragment and Aβ, both of which commonly end at the Aβ residue 40 or 42, one would expect the remaining cleavage product, APP intracellular domain (AICD), to start from either the 41\textsuperscript{st} or 43\textsuperscript{nd} residue. However, most AICDs start from the 50\textsuperscript{th} residue. This finding led to the identification of two new cleavage sites (ε and ζ sites) besides the γ-cleavage site at 42/40 that produce the final Aβ42 and Aβ40 products. In the amyloidogenic pathway, APP is first cleaved by β-secretase to produce the soluble N-terminal ectodomain and the 99 amino acid C-terminal domain, C99. C99 is then cleaved at the ε-cleavage site to produce Aβ49 and AICD. Aβ49 undergoes further
cleavage at the $\zeta$-cleavage site, resulting in $\beta46$, which becomes the final $\beta40$ and $\beta42$ products after $\gamma$-cleavage.

Modulation of $\alpha$-, $\beta$- and/or $\gamma$-secretase activity could regulate the production of $\beta$.

Stimulation of $\alpha$-secretase or inhibition of $\beta$- and $\gamma$-secretases would both reduce the amount of $\beta$ produced. $\beta$-secretase activity is increased in the cortex of AD patients but not the cerebellum, similar to $\beta$ deposition in the AD brain (Fukumoto et al., 2002). $\beta$-Secretase can be safely blocked by peptidomimetics and non-peptidomimetics (John et al., 2003). $\gamma$-Secretase inhibitors have been shown to reduce $\beta$ production but these inhibitors could affect the cleavage and function of other $\gamma$-secretase substrates and cause immunological and gastrointestinal toxicity (Guardia-Laguarda et al., 2010). Inhibitors with higher $\beta$-lowering potency and less side effects remain to be developed and tested.

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Figure 1. APP processing. APP cleavage via the non-amyloidogenic $\alpha$-secretase pathway and the amyloidogenic $\beta$-secretase pathway. Copied from LaFerla, 2002.
1.2.2 Fibrillogenesis

Aβ assumes multiple forms as it aggregates and deposits in the brain (Fig. 2). Aβ40 and Aβ42 in their native state adopt a random coil conformation but high concentrations and a suitable pH can cause Aβ peptides to adopt a β-sheet conformation (Simmons et al., 1994). Parallel β-sheet structures are present in both Aβ40 and Aβ42, which allows for hydrophobic side chain interactions and the formation of a hydrophobic core (Petkova et al., 2002; Antzutkin et al., 2002). Aβ in a random coil conformation causes minimal toxicity. In the β-sheet conformation, toxicity is greatly increased (Simmons et al., 1994). X-ray diffraction study has revealed β-sheet structures in Aβ fibres that are found in the human AD brain (Kirschner et al., 1986), suggesting that ordered β-sheet conformation is an intermediate structure for Aβ fibrillogenesis. Also β-sheet breakers, short proline containing peptides, can inhibit fibrillogenesis and disassemble fibrils (Soto et al., 1998), further demonstrating the importance of this structural transition.

Aβ aggregation starts with monomeric Aβ and is a nucleation-dependent process. The nucleus formation is a thermodynamically unfavourable step so there is a lag time before aggregates are detected; the length of this lag depends on Aβ concentration (Jarrett and Lansbury, 1993). Trace amounts of dimers and trimers are formed from monomers (Jarrett and Lansbury, 1993). As the nucleus forms, it presents multiple sites for monomer attachment, leading to thermodynamically favourable growth of the aggregate (Jarret and Landbury, 1993). Thus arising from monomers, Aβ aggregates to form various sizes of oligomers with an average size of 24 monomers long (Glabe et al., 2005) (Fig. 2). Spherical oligomers with micelle characteristics further aggregate to form protofibrils (Glabe et al., 2005). Protofibrils then undergo a conformation change to mature into fibrils (Glabe et al., 2005) (Fig. 2). Protofibrils exist in equilibrium with lower
molecular weight Aβ and share tinctorial properties with Aβ fibrils. Due to the presence of extensive β-sheet structures, Aβ can bind Congo red and Thioflavin T. Unlike low molecular weight Aβ, which does not bind to either dye, Aβ protofibrils and fibrils bind both dyes in a concentration dependent manner (Walsh et al., 1999).

![Image of Aβ Aggregation Pathway]

**Figure 2. Aβ Aggregation Pathway.** Aβ aggregation begins as Aβ monomers bind to each other to form dimers, trimers and larger oligomers. These aggregates then elongate into longer and wider amyloid protofibril. Further lateral aggregation creates Aβ fibres, which deposit into plaques.

### 1.2.3 Aβ Species and Toxicity

The amyloid hypothesis, which proposes that Aβ deposition is a causative agent of Alzheimer’s disease pathology, was first formalized in 1992 (Hardy and Higgins, 1992). In accordance with the hypothesis, aggregated Aβ, but not Aβ monomers, cause neurotoxicity *in vitro* (Loo et al., 1993). More specifically, Aβ fibrils, the aggregated Aβ structure found in compact plaques, were found to be neurotoxic (Lorenzo and Yankner, 1994). However, plaque quantity and location don’t correlate well with dementia severity (Terry et al., 1981; Dickson et al., 1995).
Further research on Aβ-induced neurotoxicity revealed several smaller species of soluble Aβ aggregates as toxic Aβ species that cause memory deficits (Lue et al., 1999; Mucke et al., 2000). Synthetic Aβ42-derived small diffusible Aβ oligomers, known as Aβ-derived diffusible ligands (ADDLs) are not fibrillar, but are globular. ADDLs have been found to disrupt long-term potentiation (LTP) in rat hippocampal slices and to cause mature neuronal death in organotypic brain slice cultures (Lambert et al., 1998). ADDLs also exhibit regional targeting of neurons. They selectively kill hippocampal neurons but not cerebellar neurons (Klein et al., 2001). These globular oligomers were also extracted from brains from APP transgenic mice and AD patients (Gong et al., 2003).

Another Aβ oligomeric species is a soluble 56kDa Aβ peptide, Aβ*56. Aβ*56 is a dodecamer purified from the brains of Tg2576 APP transgenic mice at an age when these mice exhibit memory deficits. Aβ*56 levels were inversely correlated with spatial memory in Tg2576 mice (Lesne et al., 2006). After infusing Aβ*56 into rat brains through a cannula, these rats showed impaired spatial memory compared to control (Lesne et al., 2006).

Low-weight soluble Aβ oligomers are shown to disrupt hippocampal long-term potentiation; Aβ trimers are especially potent at this disruption (Townsend et al., 2006). Soluble Aβ oligomers also interfere with complex learned behaviors and decrease dendritic spine density in the hippocampus (reviewed in Selkoe, 2008).

In AD patients, spherical Aβ assemblies, native amyloispheroids (ASPDs), have recently been isolated (Noguchi et al., 2009). ASPDs are high mass Aβ assemblies (>100kDa) that cause
neuronal damage. Unlike most other Aβ assemblies such as Aβ dimers and ADDLs that bind postsynaptically to exert toxicity, ASPDs are found presynaptically, labeled by the pre-synaptic marker bassoon (Noguchi et al., 2009).

1.2.4 Aβ42 vs. Aβ40

Aβ assembly is a nucleation-dependent process. Aβ aggregation and deposition depend on peptide seeding. Aβ peptides ending in C-terminal residue 42 act as seeds to form ordered β-sheet structures and promote Aβ fibrillogenesis. Aβ peptides ending in residue 40 alone form less ordered secondary structures unless seeded (Jarrett et al, 1993). The seeding effect of Aβ42 on Aβ aggregation is also observed in vivo. McGowan and colleagues developed transgenic mice expressing either Aβ42 (BRI-Aβ42) or Aβ40 (BRI-Aβ40) in the absence of APP overexpression. BRI-Aβ40 mice did not deposit Aβ with age, whereas BRI-Aβ42 mice exhibited parenchymal and cerebrovascular Aβ deposits by 3 month of age (McGowan et al, 2005). Aβ40 was shown to inhibit amyloid deposition in vivo. When BRI-Aβ40 was crossed with either BRI-Aβ42 mice or with Tg2576 APP overexpressing mice, Aβ deposition decreased compared to BRI-Aβ42 or Tg2576 mice alone (Kim et al, 2007). In contrast, Aβ42 accelerated Aβ deposition when BRI-Aβ42 mice were crossed with Tg2576 animals. A greater than additive increase in Aβ plaque burden, cerebral amyloid angiopathy and Aβ levels was observed in these mice (McGowan et al, 2005). Thus, determining Aβ42/Aβ40 ratio could help to predict the rate of Aβ deposition in the brain. In humans, Aβ42/Aβ40 ratio is a better biochemical marker for AD diagnosis than are Aβ42 or Aβ40 levels (Lewczuk et al, 2004). Similarly, transgenic mice with PS1 or APP mutations also show an increase in Aβ42/Aβ40 ratio or Aβ42 levels (reviewed in Golde et al, 1999). These studies show that Aβ42 is the driving force of fibrillogenesis.
1.3 Neuroinflammation

Neuroinflammation has been linked to several neurodegenerative diseases including amyotrophic lateral sclerosis (McGeer et al., 2002), Parkinson’s disease (Imamura et al., 2003) and AD (McGeer et al., 1987). In AD, Aβ causes neurotoxicity by direct interactions with neurons as discussed previously. It also recruits and activates microglia to cause indirect damage (Qin et al., 2001). Microglial activation in the brain has long been associated with AD pathogenesis (McGeer et al., 1987; Rogers et al., 1988). Microglial activation was observed in the cortex of mild AD cases, suggesting that it is an early pathogenic event (Cagnin et al., 2001). Aβ activation of microglia has been shown to cause the release of nitric oxide (Li et al., 1996), TNF-α (Dheen et al., 2004) and superoxides (Qin et al., 2002). Cytokines and chemokines secreted by microglia and astrocytes in the central nervous system modulate the inflammatory status in the brain. Cytokines act in conjunction with one another to elicit inflammatory responses. Cytokines that have been studied in relation to AD are listed in Table 1. Chemokines modulate leukocyte entry into the central nervous system in health and disease, particularly in neuroinflammation (Ubogu et al., 2006). Chemokines associated with AD are listed in Table 2.
# Table 1. Cytokines and chemokines associated with Alzheimer’s disease

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Main producer</th>
<th>Receptors</th>
<th>Effect/action</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>Microglia, Astrocytes, Perivascular macrophages, Endothelial cells</td>
<td>CD121a (IL-1RI), CD121b (IL-1RII)</td>
<td>Fever, T-cell activation, macrophage activation (Dinarello et al., 1996), elevated in the AD brain (Cacabelos et al., 1994); promotes synthesis of APP (Yang et al., 1998)</td>
</tr>
<tr>
<td>IL-6</td>
<td>Microglia, Astrocytes, Neurons, Endothelial cells, T cells, Macrophages</td>
<td>CD126, CD130</td>
<td>T- and B-cells growth and differentiation, acute phase protein production, fever (Heinrich et al., 2003; Kishimoto, 1989), associated with diffuse plaques in AD (Hull et al., 1996), neuronal differentiation (Satoh et al., 1988)</td>
</tr>
<tr>
<td>IL-12</td>
<td>Microglia, Astrocytes, Macrophages, Dendritic cells</td>
<td>IL-12Rβ1c+IL-12Rβ2</td>
<td>Activates NK cells, induces CD4 T-cell differentiation into Th1-like cells, pro-inflammatory cytokine secretion, increases production of TNF-α and IFN-γ (Trinchieri, 2003), reduced in CSF of AD patients (Rentzos et al., 2006)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Microglia, Astrocytes, Macrophages, Dendritic cells</td>
<td>TNFR1 (p55), TNFR2 (p75)</td>
<td>Induces changes in vascular endothelium, changes in cell-cell junctions with increased fluid loss, local blood clotting (Baud and Karin, 2001; Locksley et al., 2001), elevated in the AD brain (Tarkowski et al., 1999), stimulates APP cleavage by γ-secretase (Liao et al., 2004)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Endothelial cells, T cells, NK cells</td>
<td>CD119, IFNGR2</td>
<td>Macrophage and glia cell activation, increased expression of MHC molecules and antigen processing, suppresses Th2 (Schroder et al., 2003)</td>
</tr>
<tr>
<td>IL-10</td>
<td>Microglia, Monocytes</td>
<td>IL-10Rα, IL-10Rβc (IL-10R2)</td>
<td>Potent suppressant of macrophage functions, inhibits synthesis of proinflammatory cytokines such as TNF-α and IFN-γ (Moore et al., 2001)</td>
</tr>
<tr>
<td>IL-4</td>
<td>Microglia, T cells, Mast cells</td>
<td>CD124, CD132</td>
<td>B-cell activation, induces differentiation into Th2 cells (Gordon, 2003)</td>
</tr>
<tr>
<td>Chemokine</td>
<td>Main producer</td>
<td>Receptors</td>
<td>Cells attracted</td>
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<tr>
<td>CXCL8 (IL-8)</td>
<td>Monocytes</td>
<td>CXCR1</td>
<td>Neutrophils</td>
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<td></td>
<td>Macrophages</td>
<td>CXCR2</td>
<td>Naïve T cells</td>
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<td>Fibroblasts</td>
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<td></td>
<td>Endothelial cells</td>
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<tr>
<td>CXCL1 (GROα)</td>
<td>Monocytes</td>
<td>CXCR2</td>
<td>Neutrophils</td>
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<td>Fibroblasts</td>
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<td>Naïve T cells</td>
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<td></td>
<td>Endothelium</td>
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<td>Fibroblasts</td>
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<tr>
<td>CCL3 (MIP-1α)</td>
<td>Microglia</td>
<td>CCR1, 3, 5</td>
<td>Monocytes</td>
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<td>Monocytes</td>
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<td>NK and T cells</td>
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<td>Basophils</td>
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<td>Mast cells</td>
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<td>Dendritic cells</td>
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<td></td>
<td>Fibroblasts</td>
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<tr>
<td>CCL4 (MIP-1β)</td>
<td>Microglia</td>
<td>CCR1, 3, 5</td>
<td>Monocytes</td>
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<td>Neutrophils</td>
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<td></td>
<td>Endothelium</td>
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<tr>
<td>CCL2 (MCP-1)</td>
<td>Microglia</td>
<td>CCR2B</td>
<td>Monocytes</td>
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<td>Monocytes</td>
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<td>Keratinocytes</td>
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Table 2. Chemokines associated with Alzheimer’s disease
The immune response in AD activates resident brain immune cells and triggers an innate immune response. Innate immunity is a more primitive immune response compared to adaptive immunity. Innate immunity is the first line of host defense against pathogens. It employs pattern-recognition receptors including toll-like receptors (TLRs) at the cell surface or membrane of lysosomes and endosomes. In the cytoplasm, nucleotide-binding oligomerization domain-leucine-rich-repeat proteins and caspase recruitment domain-helicase proteins are used to recognize pathogens and mediate defense through phagocytosis (reviewed in Akira et al., 2006). Adaptive immunity involves gene rearrangements to produce high-affinity antigen-specific receptors and the process can take weeks. The antigen becomes either neutralized by the antibody or destroyed by cytotoxic T cells. Adaptive immunity is more specific, but innate immunity is capable of immediate response to danger (reviewed in Bhat and Steinman, 2009). In AD, an innate immune response is triggered, mediated by toll-like receptors and CD14 (Liu et al., 2005; Tahara et al., 2006).

Mediators of the innate immune system are involved in AD. The microglial receptor CD14 closely associates with Aβ42 and facilitates Aβ phagocytosis (Liu et al, 2005). Furthermore, CD14 shows pronounced immunoreactivity in microglia at lesion sites and expression is upregulated in AD patients (Liu et al., 2005; Combarros et al., 2005). Polymorphism of CD14 has been linked to increased risk for developing AD (Combarros et al., 2005). Toll-like receptors (TLRs) are also implicated in AD. Similar to CD14, activation of TLR2, TLR4 and TLR9 receptors cause Aβ phagocytosis by microglia (Richard et al., 2008; Tahara et al., 2006). Expression of toll-like receptors is down-regulated in macrophages from AD patients upon Aβ stimulation (Fiala et al., 2007).
1.3.1 Role of Microglia

Microglia are resident brain immune cells of myeloid origin and comprise about 12% of all cells in the brain (Del Rio-Hortega, 1932). Microglia survey the brain for pathogens and mostly exist in a resting state characterized by a ramified morphology (Nimmerjahn et al., 2005). When activated by a stimulus, microglia transform into an amoeboid morphology and express an array of surface markers (Cho et al., 2006). Microglia have many functions in the brain. They are involved in innate immunity (Jack et al., 2005; Town et al., 2005), programmed cell death (Upender et al., 1999; Marin-Teva et al., 2004), and also regulate neuronal survival through the release of neurotrophic factors and anti-inflammatory cytokines (Morgan et al., 2004; Aarum et al., 2003). Microglia are not evenly distributed within the CNS of humans (Mittelbronn et al., 2001) or mice (Lawson et al., 1990). In adult mice, the highest density of microglia is observed in the grey matter and particularly in the hippocampal dentate gyrus, olfactory tubercle and the basal ganglia (Lawson et al., 1990). Microglia are capable of producing many cytokines in the AD brain, including pro-inflammatory cytokines IL-1, IL-6, TNF-α and MCP-1. These cytokines have controversial roles of either being neurotoxic or neuroprotective.

1.3.2 Role of Astrocytes

The astrocyte is another type of glia cell present in the CNS. Astrocytes play essential roles in providing support for neurons. Astrocytes maintain ionic homeostasis, secrete neurotrophic factors locally for neurons, buffer neurotransmitters and are an important component of the blood-brain barrier (Aloisi et al., 1999; Hansson and Ronnback, 1995; Vernadakis et al., 1988). Activated astrocytes become hypertrophic, a state characterized by increased expression of glial
fibrillary acid protein (GFAP). In AD, astrocytes are capable of clearing Aβ by internalizing it. The clearing of Aβ is thought to be matrix metalloproteinase-9 dependent (Coray et al., 2006; Yan et al., 2006). However, plaque-associated astrocytes also extend their processes to cover the neuritic layer, possibly creating a barrier to microglial clearance of plaques (Mrak et al, 1996). Cultures containing both microglia and astrocytes showed decreased Aβ phagocytosis compared to cultures containing microglia alone (DeWitt et al., 1998). Astrocytes deposit astrocyte-derived proteoglycan, which may contribute to the inability of microglia to clear Aβ (Shaffer et al., 1995). This is consistent with the observation that proteoglycans preferentially localize to neuritic plaques (Snow et al., 1988). In addition to effects on microglial activity, astrocytes express inflammatory mediators such as IL-1, IL-6 (Vandenabeele et al., 1991; Del Bo et al., 1995) and inducible nitric oxide synthase (iNOS) (Lee et al., 1993).

### 1.3.3 IL-1β

IL-1β is a cytokine that is involved in inducing fever and in driving the expression of other cytokines to cause inflammation (Dinarello, 2009). The effect of IL-1β has been extensively studied in AD (Craft et al., 2005; Das et al., 2006; Jimenez et al., 2008; Shaftel et al., 2007). Transgenic mouse lines were made to determine the effect of up or down regulation of IL-1β in AD. In one model, inducible IL-1β over-expressing transgenic mice were crossed with APP/PS1 transgenic mice that show plaque deposition at 4 months (Shaftel et al., 2007). Mice were characterized at 7 month, immediately after turning on IL-1β overexpression for 4 weeks. Surprisingly, the mice showed a decrease in the area and number of plaques in the hippocampus as well as a decrease in insoluble Aβ40 and Aβ42 but soluble Aβ levels remained unchanged (Shaftel et al., 2007). Another study involved increasing IL-1β signaling by genetic ablation of
the endogenous IL-1 receptor antagonist. These mice exhibited higher IL-1β and TNFα levels and more microglia in the hippocampus compared to wildtype mice in response to oligomeric Aβ infusion. Levels of the post synaptic marker, PSD-95, were lower in the Aβ-infused transgenic mice and no changes in astrocyte number were observed (Craft et al, 2005). Since IL-1β over-expression did not reduce soluble Aβ in the first study (Shaftel et al., 2007), and high levels of soluble oligomeric Aβ coincided with appearance of pro-inflammatory cytokine secreting microglia (Jimenez et al., 2008), it is not surprising that inflammation and synaptic damage persisted in the IL-1 receptor antagonist knockout model of enhanced IL-1β signaling. In a model of reduced IL-1β signaling where IL-1 receptor 1 (IL-1 R1) knockout mice were crossed with APP over-expressing Tg2576 mice, no difference was observed in insoluble or soluble Aβ levels in APP/IL1- R1 -/- and APP/IL-1 R1 +/+ mice. Similarly, no difference in microglial immunoreactivity surrounding Aβ plaques was observed in these two genotypes (Das et al, 2006). Based on these studies, it is unclear whether IL-1β has beneficial or detrimental effects on the development of AD.

1.3.4 TNF-α

TNF-α is another pro-inflammatory cytokine that is elevated in the serum, CSF and brain of AD patients (Fillit et al., 1991; Tarkowski et al., 1999). Both neurotoxic and neuroprotective actions of TNFα have been reported. In transgenic mice that over-express TNF-α, driven with a GFAP or neuronal promoter, severe inflammation and neurodegeneration have been observed (Akassoglou et al., 1997; Probert et al., 1995). Similarly, TNF receptor 1 (TNFR1) knockout mice showed decreased Aβ production and plaque formation (He et al., 2007). TNFR1 also regulates β-secretase 1 (BACE1) promoter activity through NF-κB signaling, and deletion of
TNFR1 reduced BACE1 levels (Ping et al., 1997). Therefore, TNF-α signaling increases BACE1 activity and promotes amyloidogenic cleavage of APP.

In contrast, exposure of rat hippocampal neurons to TNF-α resulted in decreased peroxide accumulation and decreased glutamate excitotoxicity in response to Aβ (Barger et al., 1995). TNF-α has also been shown to induce the expression of the anti-oxidant enzyme manganese-superoxide dismutase (Keller et al., 1998). These findings indicate a neuroprotective role in AD.

The physiological response elicited by elevated TNF-α levels may depend on which receptor TNF-α binds to. TNF-α has two receptors, the p55 and p75 receptors. The p55 TNF receptor causes cell death upon activation through its intracellular death domain (Mattson et al., 1997); whereas, the p75 TNF receptor has a neuroprotective role (McKee et al., 1998). The role of TNF-α in AD, whether detrimental or beneficial, needs to be elucidated.

1.3.5 IL-6

IL-6 is expressed by microglia, astrocyte, neurons, and endothelial cells in the CNS. This cytokine is expressed mainly during development and is expressed at a low level in adults unless induced in response to pathological changes (Vallieres et al., 1997). IL-6 can be induced by IL-1β and by TNF-α (Van Wagoner et al., 1989). In the AD brain, IL-6 is associated with diffuse plaques in the cortex and hippocampus but not with compact plaques (Hull et al., 1996). Transgenic mice that over-express IL-6 driven by the GFAP promoter showed decline in avoidance learning and pathological changes that implicate IL-6 in neurodegeneration (Heyser et
al., 1997). When over-expressed, IL-6 can also cause motor impairment and seizures (Campbell, 1998; Campbell et al., 1993).

Although mostly considered as an inflammatory cytokine that leads to detrimental effects in AD, studies have shown some beneficial actions of IL-6. IL-6 generates neuronal differentiation signals and it might also be essential for astrocyte differentiation (Satoh et al., 1988; Nakashima et al., 1999). IL-6 was also shown to inhibit the immune activated synthesis of TNF-α in glial culture (Crowl et al., 1991). Thus the role of IL-6 in AD progression is not well understood.

1.3.6 CXCL1

CXCL1 is a chemokine produced by a subset of neurons and astrocytes. Over-expression of CXCL1 in oligodendrocytes recruits neutrophils into the CNS and induces astrogliosis (reviewed in Bajetto et al., 2001). There are a few studies in the literature relating CXCL1 and its receptor CXCR2 to AD. In Tg2576 mice, voluntary wheel running exercise for 3 weeks stimulated both the protein and mRNA levels of CXCL1 by 1.4-1.6 fold (Parachikova et al., 2007). Using mouse primary cortical neurons, CXCL1 treatment for 15 min showed strong activation of the extracellular receptor kinase 1/2 (ERK1/2) and the phosphoinositide kinase -3-Akt (PI-3K-Akt) pathways (Xia and Hyman 2002). As ERK1/2 (Bonni et al 1999, Kornhauser 1997) and PI-3 (Chan et al 1999) are major pathways involved in the signaling of neurotrophic factors, CXCL1 might confer neuroprotection. In another study, CXCL1 reduced cell apoptosis in Aβ42 induced cell death in primary hippocampal neurons (Watson and Fan, 2005). The neuroprotective effects of all three ligands activate both the ERK1/2 and PI3K/Akt pathways and inhibitors of either pathway attenuated neuroprotective effects (Watson and Fan, 2005).
In contrast, CXCL1 also induced tau hyper-phosphorylation in mouse primary cortical neurons (Xia and Hyman 2002). ERK1/2 is one of many kinases that have been implicated in tau phosphorylation (Hyman 1994; Trojanowski 1994; trojanowski 1993). The CXCL1 receptor CXCR2 is highly expressed in a subset of neurons in the brain (Horuk et al, 1997) and it is present in dystrophic neurites surrounding plaques (Horuk et al, 1997; Xia et al, 1997). CXCR2 is also shown to be involved in APP processing. Selective CXCR2 receptor antagonist and allosteric inhibitor of CXCR2 were both effective at blocking Aβ production. Stimulation of CXCR2 by CXCL1 and CXCL8 increased Aβ production (Bakshi et al, 2008). CXCR2 modulation of APP processing is most likely mediated through γ-secretase inhibition because CXCR2 receptor antagonist causes the accumulation of γ-secretase substrates C99 and C83 fragments of APP (Bakshi et al, 2008).

Cytokine and chemokine levels are altered in AD, changing the inflammatory status of the brain. As discussed, cytokines and chemokines have dual functions of either being neuroprotective or neurotoxic depending on receptor binding and levels in the brain. Modulation of cytokine and chemokine levels could be beneficial in ameliorating AD. As many studies have linked Aβ and neuroinflammation, modulating Aβ levels may affect cytokine and chemokine levels. Which cytokine and chemokine expressions are affected in response to Aβ accumulation or specific Aβ species requires further investigation.

1.4 Transgenic Mouse Models

A variety of transgenic mouse models have been made to exhibit phenotypes of AD. Mutations found in familial AD cases have been used to create these mouse models. Numerous models
utilize mutated human APP to induce over-production of Aβ; some APP mutations preferentially increase the production of Aβ42, the more fibrillogenic Aβ peptide, over Aβ40 (Reviewed in Golde et al., 2000). Mutations in PS1 and PS2 also cause a preferential production of Aβ42 over Aβ40 as well as neuronal and synaptic loss (Elder et al., 2010). In transgenic mouse lines harboring both mutated APP and PS1, Aβ plaque pathology becomes more extensive with an earlier onset (Elder et al., 2010). A triple transgenic model contain the Swedish APP, PS1 and P301L tau mutations exhibit amyloid plaques, neurofibrillary tangle, synaptic dysfunction and intraneuronal Aβ (Odde et al., 2003). Through studies with transgenic mice, as in humans, a link between Aβ and neuroinflammation was observed.

There have been many implications that Aβ deposition and neuroinflammation are associated in AD. Microglia from old PS1/APP transgenic mice express higher IL-1β and TNF-α levels but showed reduced levels of Aβ binding receptors, scavenger receptor A, CD36, receptor for advanced-glycosylation endproducts, and reduced Aβ degrading enzymes, insulydin, neprilysin and MMP9 compared to wild-type controls (Hickman, et al., 2008). In another PS1/APP mouse model, microglia switches from an alternatively activated state at the beginning of Aβ pathology to a classically activated state as pathology progresses, coinciding with increased accumulation of oligomeric Aβ (Jimenez, et al., 2008). Microglia in the alternatively activated state, expressing the marker YM-1, infiltrated and surrounded Aβ plaques and exhibited Aβ phagocytosis. Microglia in the classically activated state express pro-inflammatory factors and are cytotoxic. In this study, classically activated TNF-α positive microglia were observed in aged PS1/APP mice and these microglia were mainly non-plaque associated (Jimenez, et al., 2008).
Proinflammatory cytokine IL-1 is shown to up-regulate APP mRNA by binding to the APP promoter in human umbilical vein endothelial cells (Goldgaber et al., 1989). In other studies, IL-1 stimulated the activity of APP promoter in PC12 cells (Lahiri, et al. 1995) and primary rat hippocampal neurons (Yang, et al., 1998). In addition, each of the pro-inflammatory cytokines IFN-γ, IL-1β, and TNF-α are able to stimulate γ-secretase activity and increase Aβ40 levels in T20 cells (Liao, et al., 2004). Stimulation of γ-secretase activity appears to be mediated through the c-Jun N-terminal kinase-dependent MAP kinase pathway (Liao, et al., 2004). In Tg2576 mouse model of AD, exercise caused a reduction in IL-1β and TNF-α to levels comparable to wild-type mice as well as a reduction in soluble Aβ40 and soluble fibrillar Aβ (Nichol, et al., 2008).

1.4.1 TgCRND8 Mice

The TgCRND8 transgenic mouse model has been used to perform the studies in this thesis. TgCRND8 is a transgenic mouse model of Alzheimer’s disease that over-expresses the mutated human amyloid precursor protein (APP). The transgene encodes the familial Indiana and Swedish mutations on the APP gene under the control of the hamster prion (HaPrP) promoter (Chishti et al., 2001). The Swedish mutation favors β-secretase cleavage of APP and the Indiana mutation increases Aβ42/Aβ40 ratio (Mucke et al., 2000). Transgene expression is driven by the hamster prion promoter. Relatively high expression of the promoter is seen in the cerebellum, hippocampus and cerebral cortex (Race et al., 2005).
The TgCRND8 is a rapid-onset transgenic model of Alzheimer’s disease where Aβ plaques appear at 3 months of age (Chishti et al., 2001). Aβ levels did not differ between males and females in this model. Aβ levels remain low and steady for the first 10 weeks of life but increases profoundly between 10 and 26 weeks (Chishti et al., 2001). Neuronal degeneration was also evident as NeuN staining was less intense in Tg animals versus wt animals and no staining was found around Aβ42 deposits (Bellucci et al., 2006). Basal forebrain cholinergic neurons also suffered a 30% reduction by 7 months of age (Bellucci et al., 2006).

In TgCRND8 mice, there is evidence of neuroinflammation. At 7 months of age, activated microglia and hypertrophic astrocytes surround Aβ (1-42) plaques in plaque-rich regions of the brain such as the cortex, hippocampus, thalamus and the basal forebrain (Bellucci et al., 2006). Inducible nitric oxide synthase (iNOS) positive cells were also found in these brain regions (Bellucci et al., 2006). The recruitment of activated microglia and astrocytes is consistent with a previous study that showed concurrent appearance of microglia with plaques, shortly followed by the appearance of astrocytes, at 13-14 weeks of age (Dudal et al., 2004). In another study, pro-inflammatory cytokines IL-1β and TNFα, measured by ELISA, were also elevated in the brains of TgCRND8 mice compared to non-Tg controls (Chauhan et al., 2004). This is a very suitable model to study the association between Aβ accumulation and neuroinflammation in AD for a few reasons. The TgCRND8 mouse model demonstrates increasing levels of macroscopic Aβ in the mouse brain, as seen in AD patients. This mouse model also exhibits neuroinflammation, marked by activated microglia and astrocytes as well as an increase in inflammatory mediators. The extensive usage of this model has allowed it to be well characterized. The advantage of using the TgCRND8 model versus another well-characterized APP transgenic model Tg2576, which harbours the Swedish APP mutation, is that the
TgCRND8 model has both the Swedish and Indiana APP mutation. The added APP mutation allows for earlier Aβ deposition in the TgCRND8 brain, which helps to dissociate the effects of ageing from the effects caused by Aβ deposition.
Chapter 2
Hypothesis and Specific Aims

Hypothesis
Aβ deposition pattern in the brain elicits region-specific inflammatory responses.

Specific Aims
1) Aβ aggregation is not uniform across the AD brain. To determine the effect of Aβ on neuroinflammation, brain regional Aβ accumulation needs to be determined to establish the distribution of Aβ and associated inflammation. Therefore, in TgCRND8 mice, Aβ accumulation as soluble and insoluble Aβ40 and Aβ 42 will be determined in distinct brain regions as Aβ pathology develops.

2) Factors that contribute to differences in brain regional aggregation of Aβ may contribute to preferential Aβ accumulation and changes in inflammatory status. Parameters that may contribute to specific regional response to Aβ aggregation and trigger neuroinflammation will be examined. These include Aβ42/Aβ40 ratio, insoluble Aβ/soluble Aβ ratio, Aβ oligomer levels and plaques.

3) In response to Aβ levels and different Aβ species found in each region, neuroinflammation in these regions will be examined to reveal changes in inflammatory status. This will be achieved by measuring regional cytokine levels as Aβ pathology progresses.
Chapter 3
Methods

3.1 Animals

TgCRND8 mice expressing human APP with pathogenic Swedish (KM670/671NL) and Indiana (V717F) mutations under the control of Syrian hamster prion promoter were studied (Chishti, 2001). These mice were maintained on an outbred C3H/C57BL6 background and were kept on a 12 hour light/dark cycle with food and water ad libitum. Mice were sacrificed at 16, 28 or 36 weeks of age by phenobarbital anesthesia followed by intracardial perfusion with phosphate-buffered saline (PBS) and hepalean (Wyeth, Canada). All experiments were performed in accordance with Canadian Council for Animal Care and University of Toronto guidelines.

3.2 Brain dissections

Following intracardial perfusion, fresh brain dissections were performed on a chilled 4°C surface in PBS. Dissected tissues were flash frozen on dry ice and stored at -80°C. Regions dissected include the olfactory bulb, cortex, hippocampus, septum, striatum, and the cerebellum. These tissues were used for Aβ ELISAs, cytokine ELISAs and 7-plex assays.

3.3 Brain Fixation and Sectioning

Immediately following intracardial perfusion with PBS for exsanguination, PBS was replaced by 4% paraformaldehyde to perfusion fix all tissues. After fixation, brains were
removed, washed in PBS for one day and stored in 30% sucrose at 4°C. Brains were
sectioned coronally with a cyrostat at 20µm and frozen tissue sections were stored at -20°C
in anti-freeze solution (3 glycerol : 3 ethylene glycol : 4 PBS). Tissue sections were used for
immunofluorescent staining.

3.4 Aβ ELISA

Regional brain tissues from both hemispheres at 16 weeks and from one hemisphere at 28 and 36
weeks were used for Aβ ELISA sample preparation. All tissues were homogenized in sucrose
buffer, 20 mM Tris pH 7.4, 0.25 M sucrose, 1 mM EDTA, and 1 mM EGTA. Total Aβ was
extracted after sonicating homogenized tissue for 30 s in cold 70% formic acid. Soluble Aβ was
extracted from tissue homogenates in 0.4% diethylamine and 100 mM NaCl. All samples were
centrifuged at 100,000 x g for 1h at 4°C followed by neutralization of the supernatant. The total
Aβ fraction was neutralized with 1 M Tris and 0.5 M Na₂HPO₄ (19x supernatant volume) and the
soluble fraction was neutralized with 0.5 M Tris (pH 6.8) (0.1x supernatant volume). Samples
were aliquoted and stored at -80 °C. Aβ40 and Aβ42 levels total and soluble levels were
measured in triplicate with commercially available sandwich ELISA kits (BioSource, Burlington,
ON, Canada). Insoluble Aβ levels were determined by subtracting soluble Aβ levels from total
Aβ levels. Aβ levels for each sample were normalized to protein concentration in the sucrose
buffer, as determined by a BCA assay (Thermo Scientific, Rockford, IL).
3.5 Aβ Immunohistochemistry

Brain hemispheres were fixed in 4% paraformaldehyde and embedded in paraffin wax. Sagittal sections of 6 µm were deparaffinized in xylene and ethanol, then blocked with 3% hydrogen peroxide in methanol. Antigen retrieval for Aβ was performed by treating sections with 95% formic acid for 5 min. Sections were then washed in tap water and TBS to remove the formic acid and blocked with 15% goat serum. Primary antibody 6F/3D (1:400, DakoCytomation, Denmark) was applied to all sections overnight at 4°C. Sections were then washed in TBS and incubated in biotinylated anti-mouse IgG followed by streptavidin/horse radish peroxidase treatment with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Staining with visualized with diaminobenzidine and counterstained Harris hematoxylin (Sigma). Digital micrographs of brain regions were captured with a Coolsnap digital camera (Photometrics, Tuscon, AZ) mounted on Zeiss Axioscope 2 Plus microscope at 40x.

3.6 Aβ Oligomer Dot Blot

ELISA samples used to determine soluble Aβ levels were also used to analyze oligomer levels. Protein concentrations were measured by a BCA protein assay (Thermo Scientific). One gram of protein from each sample was dotted onto nitrocellulose membrane that was blocked with 10% milk in TBS-T and incubated with anti-oligomer A11 antibody (Invitrogen) in 3% BSA in TBS-T (0.01% Tween 20) overnight. The A11 antibody was biotinylated with a commercially available EZ-Link Micro Sulfo-NHS-LC-biotinylation kit (Thermo Scientific) prior to incubation. Biotinylation was performed as outlined in the manufacturer’s protocol. In brief, the A11 antibody was incubated with sulfo-NHS-LC-
biotin for 2 hrs on ice and was run on an equilibrated desalting column to remove excess biotin. Blots were then incubated in streptavidin/HRP (1:1000 dilution in 3% BSA in TBS-T) for one hour at room temperature and developed with an ECL kit (GE Healthcare, Buckinghashire, UK).

3.7 Cytokine ELISA Assays

To determine cytokine concentrations in the cortex, hippocampus, septum and cerebellum of 2 and 4 months old TgCRND8 and non-Tg littermates, ELISA assays were performed on snap frozen tissue samples. For each region, tissues from both hemispheres were homogenized in 200µl of homogenate buffer (RPMI1640 cell media + 1% protease inhibitor (Calbiochem, CA), sonicated for 30 s and centrifuged at 4 °C at 12,000 rpm for 10 min. Sample supernatants were loaded in triplicate onto commercially available sandwich ELISA kits (R & D Systems). Cytokine levels were normalized to total protein concentrations. Kits used include those produced for IL-1β, TNF-α, IL-6, IL-4 and IL-10.

3.8 7-plex Cytokine Concentration Assay

To determine cytokine peptide concentrations, 100µl of sample buffer was added to the olfactory bulb(s), septum, striatum, hippocampus, and cerebellum samples whereas 200µl sample buffer was added to the cortex. Samples were homogenized in lysis buffer (150mM NaCl, 20mM Tris, 1mM EDTA, 1mM EGTA, 1% Triton x-100) containing 1% protease inhibitor cocktail III (Calbiochem), 1% phosphatase inhibitor cocktail I (Sigma P-2850), 1% phosphatase inhibitor cocktail 2 (Sigma P-5726) and 1% BSA. Samples were sonicated
twice for 15 s, incubated on ice for 30 min and centrifuged at 15,000 rpm for 10 min at 4 °C. Supernatants were used in the assay as described in the manufacturer's protocol (Mesoscale Discovery mouse pro-inflammatory 7-plex). To ensure even distribution of solutions, plates were placed on an orbital shaker at 180rpm and incubation with the detection antibody was extended to 3 hrs. Samples were run in triplicates and sample cytokine concentrations were normalized to total protein concentration for the same sample.

3.9 Statistical Analysis

One-way ANOVA followed by the post hoc Tukey’s test was used to assess regional differences in Aβ levels at each age group investigated. Outliers were removed using Grubb’s test prior to ANOVA analysis. To assess differences in cytokine levels between TgCRND8 and wt mice, 2-tailed independent-sample Student’s t-test was used. Significance level was set to α=0.05 and all analyses were performed using SPSS. Data are presented as means ± SEM.
Chapter 4
Results

4.1 Beta-amyloid

Examining brain Aβ deposition and inflammation may distinguish regional differences in vulnerability to AD disease pathology and may reveal underlying factors that contribute to selective regional targeting of the disease. Using APP transgenic TgCRND8 mice, brain regional levels of different Aβ species and inflammatory status were examined by Aβ ELISAs and cytokine assays. Inflammation was assessed by measuring levels of pro-inflammatory cytokines and by immunofluorescent staining to reveal their cellular distribution. Mice were sacrificed at 16, 28 and 36 weeks of age. These ages were chosen as they correspond to distinct phases in development of the murine AD-like phenotype. Aβ plaques appear at approximately 13 weeks. Thus, 16 weeks represents early Aβ pathology (Chishti et al., 2001). At 28 weeks, profuse Aβ plaques are present in the brain. By 36 weeks, the amount of Aβ in the brain represents a stage of Aβ pathology found in end-stage sporadic AD patients.

Aβ and cytokine levels were measured in six brain regions, olfactory bulb, cortex, hippocampus, septum, striatum and cerebellum. The olfactory bulb, hippocampus and cortex were chosen because these are severely affected brain regions in AD. The olfactory bulbs are affected early in AD (Christen-Zaech et al., 2003) and olfactory memory is also impaired in young Tg2576 APP transgenic mice (Guerin et al., 2009). The cortex and hippocampus are also affected early in the AD pathogenesis. Brain regions that project to areas with high density of Aβ plaques exhibit profound neurodegeneration. Thus, the septum was also chosen as an area of interest because
cholinergic neurons project from the septum to the hippocampus (Dutar et al., 1995). Aβ deposition in the striatum is detected at a later phase in AD compared to the more affected regions such as the olfactory bulb, cortex and the hippocampus (Thal et al., 2002). Aβ deposition in the cerebellum is observed typically in very late AD development (Thal et al., 2002).

To evaluate Aβ deposition and neuroinflammation, TgCRND8 and wild-type mice aged 16, 28 and 36 weeks were used. Within each age group, mice were sacrificed over a 3-day window so as to minimize variations in Aβ levels. Exsanguinated mouse brains were dissected fresh at 4°C and flash frozen on dry ice to minimize peptide degradation. Tissues used for cytokine assays were prepared on the same day as the assay to avoid additional freeze-thaw cycles. Method development on various ways of tissue preparation showed that reduced freeze-thaw cycles yielded higher detectable cytokine levels. Because cytokine levels are low in chronic inflammation (Eikelenboom et al., 2002), all precautions to preserve cytokine proteins were practiced. Protein levels of pro-inflammatory cytokines were determined in all regions to assess the level of inflammation. Pro-inflammatory cytokines examined included IL-12, IL-6, IFN-γ, TNF-α, IL-1β and CXCL1. Two anti-inflammatory cytokines, IL-10 and IL-4, were also assayed to give further insight into inflammatory status of the brain.

4.2 Soluble Aβ Levels in the TgCRND8 Brain

There are many species of soluble Aβ. These include Aβ-derived diffusible ligands (ADDLs), Aβ*56 and low-molecular-weight Aβ oligomers (Lambert et al., 1998; Lesne et al., 2006; Townsend et al., 2006). Soluble Aβ has been found to disrupt long-term potentiation and soluble
Aβ levels correlate with severity of dementia (McLean et al., 1999). Aβ42 seeds the formation of ordered β-sheet structures and accelerates Aβ deposition, whereas Aβ40 inhibits Aβ deposition (Jarrett et al., 1993; Kim et al., 2007). Soluble Aβ was extracted with 0.4% diethylamine in 100mM NaCl. Aliquots containing soluble Aβ were run on Aβ42 and Aβ40 ELISA plates in triplicate to determine levels.

At 16 weeks of age, a period of 3 weeks after Aβ plaques were first observed in TgCRND8 animals (Chishti et al., 2001), soluble Aβ42 levels were lowest in the striatum (Fig. 3A, Table 3). Soluble Aβ40 levels also appeared the lowest in the striatum but no statistical significance between any two regions was apparent (Fig. 3B, Table 3).

By 28 weeks, when regional profuse Aβ plaques were present in the brain, differences in soluble Aβ expression emerged. Soluble Aβ42 remained lowest in the striatum. Levels in the cerebellum also appeared lower than those observed in the olfactory bulb, cortex, septum and hippocampus but the differences did not achieve statistical significance (Fig. 3C). Differences in soluble Aβ40 were also observed at this age (Fig. 3D). Soluble Aβ40 levels in the striatum were found to be lower than levels in the cortex, hippocampus and septum. Cerebellar levels of Aβ40 were also significantly lower than those seen in the hippocampus (Table 3).

By 36 weeks of age, the clear differences were evident in soluble Aβ42 and Aβ40, between regions of high and low vulnerability in AD. Soluble Aβ42 levels were found to be high in the olfactory bulb and hippocampus (Fig. 3E). Levels in olfactory bulb were significantly higher than levels in the cortex, septum, striatum and cerebellum (Table 3). Aβ42 levels in the
olfactory bulb and hippocampus continued to increase between 28 and 36 weeks but remained constant in other regions. In contrast, Aβ40 levels in all regions increased between 28 and 36 weeks except the striatum. Hippocampus had accumulated the highest levels of soluble Aβ40 by 36 weeks of age (Fig. 3F). Levels in the hippocampus were significantly higher than levels in the cortex, striatum and cerebellum. Soluble Aβ deposition was the lowest in the striatum, it was significantly lower compared to the hippocampus and olfactory bulb (Table 3). Olfactory bulb and hippocampus have emerged as high soluble Aβ expressing regions whereas the cortex, striatum and cerebellum were low expressing regions. Figures 4 and 5 show the accumulation of soluble Aβ40 and Aβ42 in brain regions over time; levels of Aβ accumulation are represented by the intensity of colour in a cartoon drawing of the brain.
Figure 3. Soluble Aβ accumulation by region and age. Soluble Aβ42 levels in the TgCRND8 olfactory bulb, cortex, hippocampus, septum, striatum and cerebellum at 16 weeks, 28 weeks, and 36 weeks of age are shown in panels A, C, and E respectively. Soluble Aβ40 levels in the olfactory bulb, cortex, hippocampus, septum, striatum and cerebellum at 16 weeks, 28 weeks, and 36 weeks of age are shown in panels B, D, and F. n = 4-6 for each region and age investigated.
Table 3. Regional ANOVA comparisons of soluble Aβ levels in TgCRND8 mice.

*P*-values determined from Tuckey’s post hoc test to indicate statistically significant differences in soluble Aβ levels between regions at 16, 28 and 36 weeks

<table>
<thead>
<tr>
<th>Age</th>
<th>Region (high) vs. Region (low)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 week soluble Aβ42</td>
<td>Olfactory bulb (high) vs. striatum (low)</td>
<td>0.019</td>
</tr>
<tr>
<td>28 week soluble Aβ42</td>
<td>Olfactory bulb (high) vs. striatum (low)</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>Hippocampus (high) vs. striatum (low)</td>
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</tr>
<tr>
<td></td>
<td>Septum (high) vs. striatum (low)</td>
<td>0.014</td>
</tr>
<tr>
<td>36 week soluble Aβ42</td>
<td>Olfactory bulb (high) vs. cortex (low)</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>Olfactory bulb (high) vs. septum (low)</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>Olfactory bulb (high) vs. striatum (low)</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>Hippocampus (high) vs. striatum (low)</td>
<td>0.02</td>
</tr>
<tr>
<td>16 week soluble Aβ40 (no significance)</td>
<td>Cortex (high) vs. striatum (low)</td>
<td>0.019</td>
</tr>
<tr>
<td>28 week soluble Aβ40</td>
<td>Cortex (high) vs. striatum (low)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Hippocampus (high) vs. cerebellum (low)</td>
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</tr>
<tr>
<td></td>
<td>Septum (high) vs. striatum</td>
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<td>36 week soluble Aβ40</td>
<td>Olfactory bulb (high) vs. striatum (low)</td>
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<tr>
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<td>Cortex (low) vs. hippocampus (high)</td>
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<tr>
<td></td>
<td>Hippocampus (high) vs. striatum (low)</td>
<td>&lt;0.001</td>
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<td>Hippocampus (high) vs. cerebellum (low)</td>
<td>0.002</td>
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**Soluble Aβ 40**

Figure 4. Cartoon representation of soluble Aβ40 accumulation in various regions of the brain. The intensity of colour represents the level of Aβ; the darker the colour, the higher the Aβ level.
Figure 5. Cartoon representation of soluble Aβ42 accumulation in various regions of the brain. The intensity of colour represents the level of Aβ; the darker the colour, the higher the Aβ level.
4.3 Insoluble Aβ Accumulation in the TgCRND8 Brain

Aβ aggregates to form fibrils that eventually deposit in amyloid plaques. The fibrils found in compact plaques are neurotoxic as shown in a study with primary rat hippocampal neurons (Lorenzo and Yankner, 1994). Regional levels of insoluble Aβ were determined by subtracting levels of diethylamine-soluble Aβ from levels of total formic acid-extracted Aβ. The formic acid extracts were run on Aβ42 and Aβ40 ELISA plates in triplicate to determine levels. We found insoluble Aβ40 and Aβ42 levels to be at least 10 times higher than soluble levels in TgCRND8 mice at each age examined.

At 16 weeks of age, both insoluble Aβ42 and Aβ40 were lowest in the septum and striatum whereas higher levels were found in the olfactory bulb, cortex, hippocampus and cerebellum (Fig 6A, B). Aβ42 levels in the striatum were significantly lower than in the olfactory bulb, cortex and cerebellum (Fig. 6A, Table 4). Aβ42 levels in the septum were significantly lower than in the cortex (Table 4). Similar to Aβ42 levels, Aβ40 levels were also the lowest in the septum and striatum. Higher levels were observed in the hippocampus and cerebellum whereas highest levels of insoluble Aβ40 were deposited in the olfactory bulb and cortex (Fig. 6B). Insoluble Aβ40 levels in the striatum were significantly lower than its levels in the olfactory bulb and cortex (Table 4).

At 28 weeks of age, regions expressing high levels of Aβ42 were the same as they were at 16 weeks. These include the olfactory bulb, cortex, hippocampus and cerebellum. Regions expressing high levels of Aβ40 were cortex and hippocampus. The rate of Aβ40 deposition in
the olfactory bulb and cerebellum slowed between 16 and 28 weeks. Aβ42 levels remained lowest in the septum and striatum (Fig. 6C). Insoluble Aβ42 levels in the striatum were significantly lower than in the olfactory bulb, cortex, hippocampus and cerebellum. Insoluble Aβ42 in the septum was also significantly lower than levels in the olfactory bulb, cortex and hippocampus but not the cerebellum (Table 4). Insoluble Aβ40 levels in the olfactory bulb, septum, striatum and cerebellum were all significantly lower than insoluble Aβ40 levels in the cortex. Similarly, levels in the septum, striatum and cerebellum were significantly lower than levels in the hippocampus (Table 4). Insoluble Aβ40 levels in the olfactory bulb, septum, striatum and cerebellum did not differ significantly from one another (Fig. 6D).

At 36 weeks, brain regions that tend to be affected in AD, exhibited relatively elevated levels of insoluble Aβ. Insoluble Aβ42 levels were significantly higher in the olfactory bulb, cortex and hippocampus relative to the septum and striatum (Fig 6E, Table 4). Increases in insoluble Aβ42 levels in the cerebellum slowed between 28 and 36 weeks, making its levels comparable to that of the septum and striatum (Fig 6E). Similarly, insoluble Aβ40 accumulated in high levels in the cortex and hippocampus, significantly higher than levels found in the septum, striatum and cerebellum (Fig 6F, Table 4). Insoluble Aβ40 levels in the cortex increased 9.3 fold between 28 and 36 weeks (Fig. 6F). Cortex accumulated the most insoluble Aβ40, with levels significantly higher than the olfactory bulb and hippocampus (Table 4). Figures 7 and 8 show the accumulation of soluble Aβ40 and Aβ42 in brain regions over time as intensity of colour with a cartoon drawing of the brain.
Figure 6. Insoluble Aβ accumulation by region and age. Insoluble Aβ42 levels in the TgCRND8 olfactory bulb, cortex, hippocampus, septum, striatum and cerebellum at 16 weeks, 28 weeks, and 36 weeks of age are shown in panels A, C, and E respectively. Insoluble Aβ40 levels in the olfactory bulb, cortex, hippocampus, septum, striatum and cerebellum at 16 weeks, 28 weeks, and 36 weeks mice are shown in panels B, D, and F. n = 4-6 for each region and age investigated.
### Table 4. Regional ANOVA comparisons of insoluble Aβ levels in TgCRND8 mice.

*P*-values determined from Tuckey’s post hoc test to indicate statistically significant differences in soluble Aβ levels between regions at 16, 28 and 36 weeks

<table>
<thead>
<tr>
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<th>16 week insoluble Aβ42</th>
<th>28 week insoluble Aβ42</th>
<th>36 week insoluble Aβ42</th>
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<tr>
<td>Olfactory bulb (high) vs. striatum (low)</td>
<td><em>P</em> = 0.046</td>
<td><em>P</em> = 0.034</td>
<td><em>P</em> = 0.001</td>
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<td>Cortex (high) vs. septum (low)</td>
<td><em>P</em> = 0.018</td>
<td><em>P</em> = 0.005</td>
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<td>Cortex (high) vs. striatum (low)</td>
<td><em>P</em> = 0.003</td>
<td><em>P</em> &lt; 0.001</td>
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<td>Striatum (high) vs. cerebellum (low)</td>
<td><em>P</em> = 0.013</td>
<td><em>P</em> &lt; 0.001</td>
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<tr>
<td>Olfactory bulb (high) vs. septum (low)</td>
<td><em>P</em> = 0.032</td>
<td><em>P</em> = 0.007</td>
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<td>Cortex (high) vs. septum (low)</td>
<td><em>P</em> &lt; 0.001</td>
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<td>Cortex (high) vs. cerebellum (low)</td>
<td><em>P</em> = 0.026</td>
<td><em>P</em> = 0.009</td>
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<td>Hippocampus (high) vs. septum (low)</td>
<td><em>P</em> = 0.005</td>
<td><em>P</em> = 0.005</td>
<td><em>P</em> = 0.007</td>
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<td>Hippocampus (high) vs. striatum (low)</td>
<td><em>P</em> = 0.001</td>
<td><em>P</em> = 0.007</td>
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<td>Striatum (low) vs. cerebellum (high)</td>
<td><em>P</em> = 0.009</td>
<td><em>P</em> = 0.007</td>
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<td>Olfactory bulb (high) vs. striatum (low)</td>
<td><em>P</em> = 0.022</td>
<td><em>P</em> = 0.007</td>
<td><em>P</em> &lt; 0.001</td>
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<td>Cortex (high) vs. striatum (low)</td>
<td><em>P</em> = 0.039</td>
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<tr>
<td>Olfactory bulb (low) vs. cortex (high)</td>
<td><em>P</em> = 0.007</td>
<td><em>P</em> &lt; 0.001</td>
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<td>Cortex (high) vs. septum (low)</td>
<td><em>P</em> &lt; 0.001</td>
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<td>Cortex (high) vs. striatum (low)</td>
<td><em>P</em> &lt; 0.001</td>
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<td>Cortex (high) vs. cerebellum (low)</td>
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<td><em>P</em> = 0.001</td>
<td><em>P</em> = 0.004</td>
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<td><em>P</em> = 0.024</td>
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<tr>
<td>Hippocampus (high) vs. striatum (low)</td>
<td><em>P</em> = 0.004</td>
<td><em>P</em> = 0.024</td>
<td><em>P</em> &lt; 0.001</td>
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<tr>
<td>Hippocampus (high) vs. cerebellum (low)</td>
<td><em>P</em> = 0.024</td>
<td><em>P</em> = 0.024</td>
<td><em>P</em> &lt; 0.001</td>
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Figure 7. Cartoon representation of insoluble Aβ40 accumulation in various regions of the brain. The intensity of colour represents the level of Aβ; the darker the colour, the higher the Aβ level.
Figure 8. Cartoon representation of insoluble Aβ42 accumulation in various regions of the brain. The intensity of colour represents the level of Aβ; the darker the colour, the higher the Aβ level.
Insoluble Aβ fibrils are aggregated Aβ structures found in compact plaques. Levels of insoluble Aβ corresponded to numbers of plaques observed by Aβ plaque staining. In the TgCRND8 mouse, Aβ plaque accumulation progressed from very few at 4 months of age to profuse distribution of plaques at 8 months (Fig. 9). At 16 weeks, insoluble Aβ42 and Aβ40 in the striatum were significantly lower than the olfactory bulb and the cortex (Table 4). At 28 weeks, insoluble Aβ42 and Aβ40 levels in both the septum and striatum were significantly lower than levels in the cortex and hippocampus. Aβ42 levels in the septum and striatum were also significantly lower than levels in the olfactory bulb. The same pattern held true at 36 weeks.

In regions most affected in AD patient, olfactory bulb, cortex (Fig. 9F) and hippocampus (Fig. 9C), the most plaques were seen. These regions exhibited significantly more insoluble Aβ. In the septum and striatum (Fig. 9I), fewer plaques are observed than are encountered in the more affected regions. The lower number of plaques is consistent with lower insoluble Aβ levels in these regions. These observations suggest that insoluble Aβ deposition in a specific brain region leads to plaque formation in that region. However, this observation did not hold true for the cerebellum. In the cerebellum, despite insoluble Aβ levels comparable to those of the 36 week septum and striatum, no plaques were observed at any age investigated (Fig. 9 J-L). The anomaly cerebellum could be due to the limitation of plaque types detected by the antibody 6F/3D used in the immunohistochemistry procedure. 6F/3D is an antibody raised against 8-17 amino acid residues of the Aβ peptide and it labels extracellular compact Aβ plaques (product sheet, DakoCytomation, Denmark). Diffuse plaques are found in the molecular layer of the cerebellum in AD patients (Joachim et al., 1989). As 6F/3D does not label diffuse plaques, the presence of these could go
undetected in the cerebellum despite a large tissue load of insoluble Aβ. Intraneuronal Aβ42 has been found in both human brains and transgenic APP mice (Gouras et al., 2000; Chui et al., 1999; Oddo et al., 2003). Only extracellular Aβ was detected by 6F/3D. Thus, the high levels of insoluble Aβ in cerebellum could be, in part distributed in intraneuronal Aβ pools. Alternatively, these may be sequestered in the microglia. Microglia have been shown to internalize Aβ42 via scavenger receptors (Paresce et al., 1996). Aβ can also accumulate in astrocytes in the human AD brain (Kurt et al., 1999; Thal et al., 2000). Insoluble Aβ deposited in diffuse plaques and/or accumulated intracellularly could explain the discrepancy between the presence of insoluble Aβ and the lack of plaques in the cerebellum. Whether any or all of these factors contribute to the apparent lack of Aβ plaques in cerebellum remains to be established.
Figure 9. Regional brain Aβ plaque staining. Sagittal sections of brain were immunostained with 6F/3D antibody for Aβ plaques and counterstained with hematoxylin. Representative pictures for the hippocampus (A, B, C), cortex (D, E, F), striatum (G, H, I) and cerebellum (J, K, L) at 4, 6, and 8 months respectively are shown.
4.4 Detailed Regional Analysis of Aβ Species

To further dissect the causes of differential vulnerability to Aβ, regional Aβ42/40 ratios, Aβ42 and Aβ40 insoluble/soluble ratios, and A11-positive soluble Aβ oligomer levels were analyzed. The Aβ42/Aβ40 ratio was examined because the Aβ42/Aβ40 ratio has been found to provide a better biochemical marker for AD diagnosis than Aβ42 or Aβ40 levels alone (Lewczuk et al., 2004). Transgenic mice with PS1 or APP mutations also show an increase in Aβ42/Aβ40 ratio or Aβ42 levels (reviewed in Golde et al., 2000). As Aβ42 is more fibrillogenic than Aβ40, higher Aβ42/Aβ40 ratios may be predictive of faster Aβ deposition. Aβ42 insoluble/soluble and Aβ40 insoluble/soluble ratios, if high, may also correlate positively with vulnerability to plaque pathology. Finally, Aβ oligomer levels were examined with the conformation-specific antibody, A11, that detects soluble Aβ oligomers heavier than 40kDa (Kayed et al., 2003). As Aβ oligomer concentrations correlate better with severity of dementia than do plaques (McLean et al., 1999) and as the oligomers have been shown to disrupt LTP (Lambert et al., 1998; Lesne et al., 2006; Townsend et al., 2006), it is reasonable to posit that these levels may be highest in brain regions that exhibit extensive plaque pathology and dysfunction.

4.4.1 Olfactory bulb

The olfactory bulb is affected early in AD, even earlier than the entorhinal cortex (Kovacs et al., 2000). Olfactory dysfunction presents in AD patients as decreased odour detection and identification abilities (Doty et al., 1987). Aβ42/Aβ40 ratios in the olfactory bulb decrease with disease progression. In the TgCRND8 mouse, a ratio of 9.9 was seen at 16 weeks. This dropped to 7.4 at 28 weeks and finally to 2.2 at 36 weeks (Fig. 10A). The high initial Aβ42/Aβ40 ratio could indicate that Aβ42 is driving the aggregation of Aβ. Over time, more Aβ40 becomes
incorporated into the aggregates. Aβ40 is known to inhibit amyloid deposition *in vivo* (Kim et al, 2007). Thus, a lower Aβ42/Aβ40 ratio could slow the Aβ deposition. We found that Aβ42 insoluble/soluble ratio remained constant from 16-28 weeks but trended down between 28 and 36 weeks, although the decrease was not significant (Fig. 10B). Aβ40 insoluble/soluble ratio did not change between 16 and 36 weeks (Fig. 10C). Aβ oligomer levels in the olfactory bulb also remained comparable from 16 to 36 weeks (Fig. 10D). In sum, only the Aβ42/Aβ40 ratio appeared to contribute to Aβ deposition in the olfactory bulb.
Figure 10. Representation of Aβ species in olfactory bulb. Aβ42/Aβ40 ratios decreased from 16 to 36 weeks, $P = 0.024$ (A). Aβ42 and Aβ40 insoluble/soluble ratios (B, C) and soluble A11-positive Aβ oligomer levels (D) did not change with increased Aβ deposition.
4.4.2 Cortex

The neocortex is where Aβ first deposits in the human AD brain and especially so in the frontal, temporal and occipital lobes (Braak and Braak, 1991). The cortex is also an area of extensive Aβ42 deposition in 7-month-old TgCRND8 mice (Bellucci et al., 2006). Aβ42/Aβ40 ratios in the cortex decreased significantly from 8.6 at 16 weeks, to 3.1 at 28 weeks and to 0.6 at 36 weeks (Fig. 11A). Aβ42 and Aβ40 insoluble/soluble ratios do not change with disease progression (Fig. 11B, C). Aβ oligomer levels in the cortex also remained constant from 16 through to 36 weeks (Fig. 11D). As in to the olfactory bulb, Aβ42/Aβ40 ratios decreased, but no other Aβ parameters changed over time. Thus, the high Aβ42/Aβ40 ratio, seen at 16 weeks in this region may also drive Aβ deposition in the cortex.
Figure 11. Representation of Aβ species in cortex. Aβ42/Aβ40 ratios decreased from 16 to 28 weeks, # $P = 0.015$ and from 16 to 36 weeks * $P = 0.002$ (A). Aβ42 and Aβ40 insoluble/soluble ratios (B, C) and soluble A11-positive Aβ oligomer levels (D) did not change with increased Aβ deposition.
4.4.3 Hippocampus

Aβ deposition in the hippocampus occurs after neocortical deposition in human AD (Braak and Braak, 1991). In 7-month-old TgCRND8 mice, the hippocampus is a major site of Aβ42 deposition (Bellucci et al., 2006). Aβ42/Aβ40 ratios in the hippocampus decreased from 8.8 at 16 weeks, to 3.3 at 28 weeks and finally to 1.5 at 36 weeks (Fig. 12A). The Aβ42 insoluble/soluble ratio also decreased with advancing age (Fig. 12B). Aβ40 insoluble/soluble ratios did not change with age (Fig. 12C). The Aβ oligomer levels in hippocampus increased significantly between 16 and 28 weeks and remained constant between 28 and 36 weeks (Fig. 12D). Aβ42/Aβ40 ratios decreased as they did in the olfactory bulb and cortex. The Aβ42 insoluble/soluble ratio in the hippocampus also decreased significantly, whereas oligomeric Aβ increased in this region. As various forms of Aβ oligomers have been shown to disrupt LTP in the hippocampus as well as cause spatial memory deficit in APP transgenic mice (Lambert et al., 1998; Lesne et al., 2006; Townsend et al., 2006), the increase in oligomeric Aβ is consistent with the progressive spatial memory deficit seen in TgCRND8 mice (McLaurin et al., 2006).
Figure 12. Representation of Aβ species in hippocampus. Aβ42/Aβ40 ratios significantly decreased from 16 to 28 weeks, # P = 0.001 and from 16 to 36 weeks, * P < 0.001 (A). Aβ42 insoluble/soluble ratios also decreased from 16 to 36 weeks, * P = 0.003 and from 28 to 36 weeks, # P = 0.047 (B). Aβ40 insoluble/soluble ratios (C) did not change with increased Aβ deposition overtime. Soluble A11-positive Aβ oligomer levels increased between 16 to 28 week and remained stable until 36 weeks, # P = 0.004, * P = 0.003 (D).
4.4.4 Septum

Cholinergic neurons in the septum project to the hippocampus. The septum has been shown to express high levels of Aβ42 deposition in 7-month-old TgCRND8 mice (Bellucci et al., 2006). We found that Aβ42/Aβ40 ratios in the septum was 4.1 at 16 weeks, decreased slightly to 3.4 at 28 weeks and reached 1.8 by 36 weeks; the decrease from 16 to 36 weeks was significant (Fig. 13A). We saw no significant change in Aβ42 or Aβ40 insoluble/soluble ratios with advancing age (Fig. 13B, C). In the olfactory bulb, cortex and hippocampus, a high initial Aβ42/Aβ40 ratio most likely drives the Aβ deposition. However, in the septum, the highest Aβ42/Aβ40 ratio at 16 weeks was only approximately half of ratio found in those other regions. This could explain the slowed Aβ accumulation and fewer Aβ plaques in the septum. Aβ oligomer levels in the septum were found to be too low to measure with dot blots, even after maximum overnight film exposure and biotinylation of the anti-oligomeric A11 antibody.
Figure 13. Representation of Aβ species in septum. Aβ42/Aβ40 ratios significantly decreased from 16 to 36 weeks, $P = 0.02$ (A). Aβ42 and Aβ40 insoluble/soluble ratios (B, C) did not change with increased Aβ deposition.
4.4.5 Striatum

In clinical AD, Aβ deposition appears in the striatum at phase III of the disease, following the development of plaques in the neocortex and hippocampus at phases I and II respectively (Thal et al., 2002). Thus, one might expect Aβ levels in the striatum to be lower than those in olfactory bulb, cortex and hippocampus. Aβ42/Aβ40 ratios in the striatum of 4.8 were observed at 16 weeks. These increased to 9.2 at 28 weeks and then dropped to 3.7 at 36 weeks (Fig. 14A). The increase between 16-28 weeks and the decrease between 28-36 weeks were both significant. The rise and fall of Aβ42/Aβ40 ratios in the striatum differed from the strictly decreasing pattern observed in other regions. Insoluble/soluble ratios of both Aβ42 and Aβ40 did not change significantly from 16 to 36 weeks (Fig. 14B, C). Aβ oligomer levels in the striatum decreased from 16-28 weeks and remained low between 28 and 36 weeks (Fig. 14D). The highest Aβ42/Aβ40 ratio was achieved much later in the striatum, at 28 weeks, than at 16 weeks in other regions. This could explain why less Aβ deposits were observed in the striatum relative to other regions. Moreover, a drop in Aβ oligomer levels over time could also explain relative sparing of the striatum in TgCRND8 mouse as in clinical AD.
Figure 14. Representation of Aβ species in the striatum. Aβ42/Aβ40 ratios significantly increased from 16 to 28 weeks, $P = 0.004$ and significantly decreased from 28 to 36 weeks, $P = 0.001$ (A). Aβ42 and Aβ40 insoluble/soluble ratios (B, C) did not change with increased Aβ deposition overtime. Soluble Aβ oligomer levels decreased from 16 weeks to 28 weeks $P = 0.025$ (D).
4.4.6 Cerebellum

Aβ deposition in the cerebellum is only seen in phase 5 of AD, a late stage in the deposition process (Thal et al., 2002). Similarly, pathology appeared to be delayed in the TgCRND8 mouse. Even at 36 weeks, plaques were absent in the cerebellum. Aβ42/Aβ40 ratios decreased from 10 to 6.03 to 2.7 at 16, 28 and 36 weeks respectively (Fig. 15A). Aβ42 insoluble/soluble ratios decreased between 16 and 36 weeks, suggesting a relative increase in soluble Aβ42. Aβ40 insoluble/soluble ratios did not change over time (Fig. 15C). Aβ oligomer levels were also conserved (Fig. 15D). Despite the presence of insoluble Aβ in the cerebellum, compact plaques were not seen (Fig. 9). Insoluble Aβ can deposit extracellularly as diffuse plaques. These have been described in the cerebellum of AD patients (Joachim et al., 1989). Insoluble Aβ also accumulates intracellularly in neurons, microglia and astrocytes in humans (Gouras et al., 2000; Paresce et al., 1996; Kurt et al., 1999) and intraneuronally in transgenic mice (Oddo et al., 2003).
Figure 15. Representation of Aβ species in cerebellum. Aβ42/Aβ40 ratios decreased between 16 and 36 weeks, \( P = 0.003 \) (A). Aβ42 insoluble/soluble ratio decreased from 16 to 36 weeks, \( P = 0.01 \) (B) while the Aβ40 insoluble/soluble ratio (C), and soluble A11-positive Aβ oligomer levels (D) did not change between 16 and 36 weeks.
4.5 Summary of Aβ results

Soluble Aβ40 and Aβ42 had the highest accumulation in olfactory bulb and hippocampus. Their levels were relatively high in the septum and cortex at 28 weeks but the accumulation slowed between 28 and 36 weeks. The striatum consistently expressed the lowest levels of both soluble Aβ40 and Aβ42.

Starting at 16 weeks, insoluble Aβ40 and Aβ42 predominantly accumulated in the olfactory bulb and cortex. By 28 weeks, hippocampus also became a region of high insoluble Aβ40 and Aβ42 accumulation. The septum and striatum were regions of low insoluble Aβ accumulation. The levels of insoluble Aβ present in each region were reflected by the amount of plaques observed in that region, with the exception of cerebellum where moderate insoluble Aβ levels did not translate to the appearance of Aβ plaques. This sequence of insoluble Aβ deposition is in agreement with the Aβ deposition pattern in humans as shown by Thal et al., 2002.

Regional analysis of Aβ revealed hippocampus as the only region that showed an increase in A11-positive Aβ oligomers. As various Aβ oligomers have been shown to disrupt LTP (Lambert et al., 1998; Lesne et al., 2006; Townsend et al., 2006), elevated oligomer levels in the hippocampus could explain the spatial memory deficit associated with hippocampal dysfunction.

To further investigate regional differences, Aβ42/Aβ40 ratios were examined. Since Aβ42 is the more fibrillogenic species, a high ratio would indicate a tendency for Aβ to aggregate. In all regions investigated, Aβ42/Aβ40 ratios were highest at 16 weeks, possibly driving the aggregation process. The exception is the striatum where the ratio peaked at 28 weeks instead of
16. This could explain the low plaque levels in the striatum because the high ratio was delayed, which delayed the aggregation process. Although Aβ42/Aβ40 ratio is highest at 16 weeks in the septum, the ratio was only approximately half that in other regions. This could explain the low insoluble Aβ levels in the septum.

4.6 Neuroinflammation

Neuroinflammation is a characteristic of Alzheimer’s disease. Microglia and astrocytes become activated and associate with extracellular Aβ (Sasaki et al., 1997; Mrak et al., 1996). Activated glial cells also secrete cytokines and chemokines to cause an inflammatory response to aggregated Aβ.

Inflammation was assessed by measuring protein levels of cytokines in the olfactory bulb, cortex, hippocampus, septum, striatum, and cerebellum, in TgCRND8 and age-matched non-Tg wildtype controls (wt), at 16, 28, and 36 weeks of age. Commercial cytokine ELISAs kits (R & D Systems) were initially used to measure regional levels of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6 and the anti-inflammatory IL-4. Levels of IL-1β were near the detection limit of the kit, below the linear portion of the standard curve (data not shown). TNF-α was not detected in any of the 4 regions investigated, cortex, hippocampus, septum or cerebellum. IL-6 levels were above detection limits and values fell in the linear portion of the standard curve, as shown in Fig. 16 for 16 weeks-old mice. However, no significant differences in IL-6 levels were observed between TgCRND8 and wt mice. The ELISA kit did not detect any IL-4. Different methods of tissue preparation were experimented with in hopes of reducing cytokine degradation. Adjustments to the protocol in terms of tissue homogenization buffers, duration of
tissue sonication, speed of centrifugation and avoidance of a sample freeze-thaw cycle failed to improve detection of brain cytokine levels.

An alternative cytokine assay was therefore used. An ultra-sensitive mouse pro-inflammatory 7-plex kit, that allows for the simultaneous detection of 6 pro-inflammation cytokines IL-12, IL-6, IFN-γ, TNF-α, IL-1β and CXCL1 and one anti-inflammatory cytokine IL-10, was used to evaluate cytokine expression. This assay is more sensitive than the ELISA assays used previously.
4.7 Regional cytokine levels

With the 7-plex kit, TNF-α levels in all brain were below the detection level of the assay (data not shown). These results combined with negative findings in the ELISA experiments indicate that TNFα levels were very low in both TgCRND8 and control wt brains. We tentatively conclude that TNFα does not play a significant role in the TgCRND8 phenotype.

In both TgCRND8 and wt animals, IL-12, IL-6, IFN-γ and IL-10 levels were evaluated in all regions investigated but only levels in the cortex were detected at 16 and 28 weeks, not 36 weeks (Fig. 17). IFN-γ levels in the cortex at 16 weeks are significantly higher than their levels at 28 weeks (P < 0.05) (Fig. 17C). No detectable levels were found in all other regions investigated at any age. No difference in IL-6 levels was observed between TgCRND8 and wt cortex confirming IL-6 ELISA results.
Figure 17. Age-dependent changes in cortical cytokine levels of TgCRND8 and wt. No differences were observed in the pro-inflammatory cytokines IL-12, IL-6 or in the anti-inflammatory cytokine IL-10 (A, B, D). A significant age-dependent decrease in IFN-γ was observed between 16 and 28 weeks (*P = 0.011, #P = 0.009) (C). N.D. indicates levels below the threshold of detection.
4.8 IL-1β levels

IL-1β levels are elevated in the brains of AD patients (Cacabelos et al., 1994). Consistent with its role as an inflammatory mediator, IL-1β is expressed by both microglia and astrocytes (Pearson et al., 1999). IL-1 also up-regulates the expression of APP mRNA by binding to the APP promoter in human umbilical vein endothelial cells (Goldgaber et al., 1989) and stimulates the activity of APP promoter in PC12 cells (Lahiri, et al. 1995) and primary rat hippocampal neurons (Yang, et al., 1998).

Analysis of IL-1β protein levels revealed no differences between TgCRND8 and wt animals in any regions at 16 weeks (Fig. 18A). At 28 weeks, IL-1β levels in the cortex and hippocampus were found to be elevated in TgCRND8 mice (Fig. 18B). At 36 weeks, elevated levels of IL-1β were also seen in olfactory bulb, striatum and cortex (Fig. 18C).
Figure 18. Regional IL-1β levels in TgCRND8 and wt mice at 16, 28 and 36 weeks. There was no significant difference in IL-1β level at 16 weeks (A). At 28 weeks, IL-1β levels were significantly elevated in the cortex and hippocampus of TgCRND8 mice (B). At 36 weeks, IL-1β levels in the olfactory bulb, cortex and striatum are significantly higher in the TgCRND8 compared to wild-type mice (C).
4.9 CXCL1 levels

CXCL1 is a chemokine primarily produced by neurons and astrocytes. CXCL1 may activate neurotrophic effector pathways of ERK1/2 and PI-3K-Akt (Xia and Hyman, 2002). However, CXCL1 can also induce tau hyperphosphorylation in mouse primary cortical neurons through activation of ERK1/2-mediated regulation of tau phosphorylation (Xia and Hyman, 2002). Through activation of CXCR2, its receptor, CXCL1 can also increase Aβ production (Bakshi et al, 2008).

Significant differences in CXCL1 between TgCRND8 and wt animals were not found in 16 or 28 week old animals. However, by 36 weeks, CXCL1 levels in the olfactory bulb of TgCRND8 mice were significantly higher than those in wt mice ($P = 0.027$) (Fig. 19). CXCL1 levels also trended higher in the 36-week old Tg hippocampus and cortex (Fig. 19). These results suggested that CXCL1 may be a potential factor in progression from moderate to severe stage of the TgCRND8 phenotype.
Figure 19. Regional CXCL1 levels in TgCRND8 and wild-type mice at 16 weeks (A), 28 weeks (B) and 36 weeks (C) of age. At 36 weeks, CXCL1 levels in the olfactory bulb were significantly higher in TgCRND8 mice compared to wt littermates (C).
4.10 Summary of regional neuroinflammation

At 16 weeks of age, no significant differences were found between TgCRND8 and wt mice in any region. At 28 weeks, the 7-plex assay revealed IL-1β levels to be elevated in the cortex and hippocampus of TgCRND8 animals relative to wt. At 36 weeks, IL-1β levels in the cortex remained elevated in TgCRND8 mice along with the olfactory bulb and striatum. CXCL1 levels also become significantly elevated in the olfactory bulb in the TgCRND8 mice at 36 weeks. No significant differences of IL-12, IL-6, IFN-γ and IL-10 levels were found in any brain regions at any age between TgCRND8 and wt mice. TNF-α levels were undetectable by the assay at any age in either the TgCRND8 or the wt mice.

4.11 Regional Aβ in relation to neuroinflammation

At 28 weeks, Aβ42 levels in the olfactory bulb, cortex and hippocampus became significantly higher than the striatum and septum. Likewise, Aβ40 levels in the cortex and hippocampus become significantly higher than in the septum and striatum. The separation in Aβ deposition between high expressing regions and low expressing regions was found to be more pronounced at 36 weeks. All three regions expressing high levels of Aβ (olfactory bulb, cortex and hippocampus) are severely affected in AD. At 28 weeks, a stage in the murine phenotype when Aβ preferentially deposits, IL-1β levels became elevated in the TgCRND8 cortex and hippocampus. By 36 weeks, IL-1β levels in TgCRND8 had become elevated in more regions, including the olfactory bulb, and striatum. Aβ levels in the striatum at 36 weeks were comparable to those encountered in hippocampus and olfactory bulb at 28 weeks. Although IL-
1β levels remained elevated at 36 weeks in the TgCRND8 mice compared to wt, the difference was no longer significant.

CXCL1 levels in the TgCRND8 mice were elevated compared to wt in the olfactory bulb 36 weeks. The response of CXCL1 to Aβ deposition was delayed compared to IL-1β and its response may be region-specific as well as Aβ42-specific because only Aβ42 levels but not Aβ40 levels in the olfactory bulb was significantly higher than the striatum and septum.

Regional Aβ accumulation and associated inflammatory response was also influenced by the local environment of the region. Regional differences in vulnerability to Aβ and neuroinflammation required detailed analysis of many contributing factors in each region. There is also a temporal pattern involving the interaction of Aβ and neuroinflammation. Aβ deposits in the brain first, followed by elevated IL-1β levels at 28 weeks, then elevated CXCL1 levels at 36 weeks. This sequence of events is summarized in Table 5. The accumulation of Aβ may be a driving force that sets off a cascade of inflammatory events.
Table 5. Regional Summary of Aβ Accumulation
Followed by IL-1β then CXCL1 Elevation

Regions expressing high levels of Aβ and cytokines in 16-, 28- and 36-week old TgCRND8 mice. Regions listed as high levels of soluble and insoluble Aβ express levels significantly higher than at least one other region in the brain. Regions listed as high levels of cytokine expression express significantly higher cytokine levels in the TgCRND8 mice relative to wt littermates.

<table>
<thead>
<tr>
<th></th>
<th>High Soluble Aβ40 Regions</th>
<th>High Soluble Aβ42 Regions</th>
<th>High Insoluble Aβ40 Regions</th>
<th>High Insoluble Aβ42 Regions</th>
<th>High IL-1β Regions</th>
<th>High CXCL1 Regions</th>
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<td>olfactory bulb cortex</td>
<td>olfactory bulb cortex cerebellum</td>
<td>none</td>
<td>none</td>
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<tr>
<td></td>
<td></td>
<td>olfactory bulb bulb</td>
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<tr>
<td><strong>28 week</strong></td>
<td>cortex hippocampus</td>
<td>olfactory bulb bulb</td>
<td>olfactory bulb bulb cortex</td>
<td>olfactory bulb bulb cortex hippocampus cerebellum</td>
<td>cortex</td>
<td>none</td>
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<tr>
<td></td>
<td>septum</td>
<td>hippocampus septum</td>
<td>hippocampus</td>
<td>hippocampus</td>
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<tr>
<td><strong>36 week</strong></td>
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<td>cortex</td>
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<td>olfactory bulb bulb</td>
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Chapter 5
Discussion

The accumulation of Aβ in the brain is a hallmark of AD. Activated microglia and astrocytes are also abundant in the AD brain along with inflammatory mediators they secrete such as cytokines, chemokines and reactive oxygen species. Aβ triggers the activation of microglia and astrocytes thereby contributing to neuroinflammation. Cytokines such as IL-1β have also been shown to modulate Aβ levels in the brain. Aβ pathology is not uniform in the brain. Rather, Aβ deposition appears to target different brain regions at different time points in disease progression. In the TgCRND8 transgenic mouse model of AD regional Aβ accumulation and neuroinflammation were investigated.

Brain Aβ analysis in the TgCRND8 mouse covered three stages of Aβ pathology from shortly after initiation of Aβ deposition in plaques (16 weeks) to a stage wherein abundant plaques are seen (28 weeks) and finally to a stage wherein plaque pathology is equivalent to that seen in the end-stage human disease (36 weeks). At 28 weeks, soluble Aβ42 accumulation was most pronounced in the olfactory bulb, hippocampus and septum. By 36 weeks, the increase in soluble Aβ42 throughout the brain left only olfactory bulb and hippocampus as areas with particularly elevated levels. Soluble Aβ levels were consistently lower in the striatum than they were in other regions investigated.

Soluble Aβ levels were lower than insoluble Aβ levels in all regions and ages examined. The soluble fraction includes smaller aggregated Aβ species composed of varying numbers of Aβ monomers. A few soluble oligomeric Aβ species have been identified and studied (Lambert et
al., 1998; Lesne et al., 2006; Townsend et al., 2006). For example, ADDLs were found to disrupt LTP in rat hippocampal slices and to cause neuronal death at nanomolar concentrations, possibly by binding to cell surface proteins (Lambert et al., 1998). They appear to selectively target hippocampal neurons but not cerebellar neurons (Lambert et al., 1998). Low-n Aβ oligomers and especially trimers cause disruption of LTP (Townsend et al., 2006). Another Aβ oligomer isolated from the APP transgenic mouse is Aβ*56. Levels of Aβ*56 correlate inversely with spatial memory (Lesne et al., 2006). The appearance of Aβ*56 coincides with spatial memory impairment in Tg2576 animals. As the loss of spatial memory stabilizes so does the decline in Aβ*56 levels (Lesne et al., 2006). In humans, spherical Aβ assemblies were isolated from frozen AD brains and their toxicity was assessed in functional neuronal cells differentiated from human bone marrow stromal cells (Noguchi et al., 2009). More of these Aβ assemblies were found in the most severe AD cases, as assessed by the Consortium to Establish a Registry for Alzheimer’s Disease (Noguchi et al., 2009). Functional neuronal cells were derived from human bone marrow stromal cells using neurotrophic factors to bypass ethical and practical problems with using primary human neurons. At the cellular level, spherical Aβ assemblies caused degeneration of cells via pre-synaptic mechanisms as opposed to post-synaptic signaling mechanisms other soluble Aβ assemblies employ (Noguchi et al., 2009).

From 28 weeks, the levels of soluble Aβ in TgCRND8 mice were found to be much higher in the hippocampus than in the striatum. Similarly, levels of Aβ oligomers in the hippocampus, as detected by oligomer conformation-specific antibody A11, were significantly elevated relative to striatum both at 28 weeks and at 36 weeks. The high soluble Aβ and oligomer levels in hippocampus, and published studies that showed neurotoxicity of these oligomers in hippocampus (Lambert et al., 1998; Lesne et al., 2006; Townsend et al., 2006) may explain why
this structure is severely affected in the murine AD models. In contrast, the low levels of both soluble Aβ42 and Aβ40 in the striatum and the drop in striatal levels are consistent with the fact that the striatum is affected later than the hippocampus in AD models, as in the human disease (Braak et al., 2006).

In the TgCRND8 mice, both insoluble Aβ42 and Aβ40 deposited in the olfactory bulb and cortex, which coincided with the appearance of plaques at 16 weeks. Increased Aβ42 and Aβ40 deposition in the hippocampus followed at 28 weeks. By 36 weeks, the olfactory bulb, cortex and hippocampus were where the highest levels of insoluble Aβ42 and Aβ40 were found. The septum and striatum are regions wherein little insoluble Aβ deposits. The cerebellum exhibited high Aβ42 and Aβ40 levels at 16 weeks. However, the increase in these levels slowed with ageing and insoluble Aβ levels in 36 week-old cerebellum were below the high Aβ depositing regions.

Regional Aβ deposition in the TgCRND8 mice reflected the temporal and spatial pattern of Aβ deposition in the human brain. In humans, Aβ deposits are first observed in the frontal, parietal, temporal and occipital cortices. The entorhinal region and CA1 of the hippocampus are affected early in the disease, followed by subcortical regions such as the striatum, basal forebrain nuclei, thalamus and the hypothalamus. Only at late stages of AD is Aβ deposition observed in the brainstem and cerebellum (Thal et al., 2002). Consistent with this pattern of plaque distribution, olfactory bulb degeneration has been reported in all AD cases and severe degeneration was observed in a young FAD patient with PS1 mutation (Christen-Zaech et al., 2003). Similarly, in Tg2576 mice, olfactory memory is impaired although odour detection is normal (Guerin et al., 2009).
Both the production and clearance of Aβ contributes to the Aβ levels in the brain. There are also regional differences in production and clearance that could cause varied Aβ accumulation across the brain. In the TgCRND8 mice, the expression of hamster prion promoter, which drives the expression of APP, is highest in the hippocampus and cerebellum with moderation expression in the cortex (Race et al., 2005). The activity of β-secretase is increased in the cortex of AD patients (Fukumoto et al., 2002). High APP promoter expression and β-secretase activity both contribute increased Aβ production and aggregation. There are also regional differences in Aβ degradation. Neprilysin, an Aβ degrading enzyme, has the lowest expression in the hippocampus and highest expression in the striatum (Yasojima et al., 2001). This varied regional expression of neprilysin could help to explain the much higher Aβ accumulation in the hippocampus compared to the striatum. Another Aβ degrading enzyme, membrane-bound insulin degrading enzyme, also has reduced expression in the hippocampus in mild AD cases and the problem exacerbates as AD becomes more severe (Zhao et al., 2007).

The high level of insoluble Aβ seen in the 16 week-old cerebellum could be due to the high expression of the hamster prion promoter, that drives the expression of mutant APP in TgCRND8 mice (Race et al., 2005). In the cerebellum, moderate insoluble Aβ levels were detected but no extracellular compact plaques were observed even in 36 week-old TgCRND8 mice. Similarly, immunohistochemical analysis using the antibody 6E10 (reactive to 1-17 amino acids of the Aβ peptide) showed Aβ deposition in the hippocampus, cortex and olfactory bulb of 13.5 month old Tg2576 animals but failed to detect Aβ staining in cerebellum (Lehman et al., 2003). These Aβ deposits corresponds to Aβ40 levels measured in these regions. However, in the cerebellum, even though the Aβ40 levels are higher than the cortex, no Aβ staining is observed (Lehman et al., 2003).
Experimental paradigms may affect the visualization of insoluble Aβ deposits. The 6F/3D antibody used to visualize plaques in this study only detects extracellular compact plaques. Aβ could deposit in the cerebellum either as extracellular diffuse plaques or intracellularly. Such diffuse plaques are present in the molecular layer of the cerebellum in AD patients (Joachim et al., 1989). It is also possible that the cellular location of Aβ in the cerebellum may differ from other brain areas. Intraneuronal Aβ42 in humans likely takes a non-β-sheet conformation as it lacks thioflavin S and congo red staining (Gouras et al., 2000). While this study did not present any findings regarding the cerebellum, it is possible that similar speciation of Aβ may contribute to the lack of signal in TgCRND8 cerebellar section. Intraneuronal Aβ may yet be toxic. For example, a transgenic mouse model with a PS1 mutation showed a significantly lower number of neurons in the frontal cortex, hippocampus and the cerebellar Purkinje cells without Aβ plaque deposition (Chui et al., 1999). In this study, intracellular neuronal Aβ increased with disease progression (Chui et al., 1999). In triple transgenic mice, expressing mutant PS1, APP and tau transgenes, intracellular Aβ was also observed (Oddo et al., 2003). The authors proposed that intraneuronal Aβ is responsible for synaptic dysfunction (Oddo et al., 2003). Finally, Aβ can be found in microglia as they internalize Aβ42 via scavenger receptors (Paresce et al., 1996) as well as in astrocytes in the human AD brain (Kurt et al., 1999; Thal et al., 2000). Thus uptake into these cellular reservoirs could explain Aβ accumulation in the cerebellum in the absence of compact plaques.

To determine whether differences in Aβ42/Aβ40 might account differential regional Aβ accumulation, levels of both were compared in each region. It has been shown that Aβ42 is
more hydrophobic at the C-terminal and forms more ordered β-sheet structures compared to Aβ40 (Jarrett et al., 1993). Aβ aggregation is a nucleation-dependent process and Aβ42 can act as a seed for the aggregation. *In vivo*, Aβ40 inhibits amyloid deposition (Kim et al., 2007) whereas Aβ42 accelerates Aβ deposition (McGowan et al., 2005). Therefore, determining Aβ42/Aβ40 ratio could help to predict the rate of Aβ deposition in the brain.

We found that Aβ42/Aβ40 ratios decreased over time in all regions investigated. This is consistent with literature wherein transgenic mice with PS1 or APP mutations also showed an increase in Aβ42/Aβ40 ratio or Aβ42 levels (reviewed in Golde et al, 1999). In the septum, the highest Aβ42/Aβ40 ratio observed was only half of that observed in olfactory bulb, hippocampus and cortex. Thus the presence of comparatively less Aβ42 to drive the aggregation process could explain why the extent of Aβ pathology in the septum is less severe. The Aβ42/Aβ40 ratio reaches its highest in the striatum at 28 weeks instead of 16 weeks. This delay could have caused the low insoluble Aβ deposition in the striatum at a given age compared to other regions. Consistent with the literature (Lewczuk et al., 2004; Golde et al., 1999), Aβ42/Aβ40 levels also predict regional brain vulnerabilities to Aβ deposition in TgCRND8 mice.

There are many indications in the literature that Aβ deposition and neuroinflammation are associated in AD. Microglia from old PS1/APP transgenic mice express higher levels of IL-1β and TNF-α levels but reduced levels of the Aβ binding receptors SRA, CD36 and RAGE as well as reduced levels of the Aβ degrading enzymes insulysin, neprilysin and MMP9, compared to wild-type controls (Hickman, et al., 2008). In another PS1/APP mouse model, microglia were found to switch from an alternatively activated state at the beginning of Aβ pathology to a classically activated state as pathology progresses and increased accumulation of oligomeric Aβ.
occurs (Jimenez, et al., 2008). The proinflammatory cytokine, IL-1 was shown to up-regulate APP mRNA by binding to the APP promoter in human umbilical vein endothelial cells (Goldgaber et al., 1989). In other studies, IL-1 stimulated the activity of APP promoter in PC12 cells (Lahiri et al. 1995) and in primary rat hippocampal neurons (Yang et al., 1998). In addition, each of the pro-inflammatory cytokines IFN-γ, IL-1β, and TNF-α stimulated γ-secretase activity and increased Aβ40 levels in T20 cells (Liao et al., 2004). Stimulation of γ-secretase activity appears to be mediated through the c-Jun N-terminal kinase-dependent MAP kinase pathway (Liao et al., 2004). In the Tg2576 mouse model of AD, exercise reduced both soluble Aβ and caused a reduction in IL-1β and TNF-α levels to those of wild-type (Nichol et al., 2008).

Collectively, these data suggest that Aβ modulates the inflammatory state in the AD brain and alters microglial phenotype. Pro-inflammatory cytokines in turn stimulate APP production and γ-secretase activity, which may increase Aβ deposition in a positive feedback loop.

To evaluate the relationship between neuroinflammation and Aβ, inflammatory mediators were examined in the regional brain samples. As Aβ deposits in the brain, IL-1β protein levels increase in the cortex and hippocampus of TgCRND8 mice compared to wild-type mice at 28 weeks age. At 36 weeks of age, IL-1β levels were significantly higher in the olfactory bulb, cortex and striatum of TgCRND8 mice. Aβ levels in the striatum at 36 weeks were comparable to Aβ levels at 28 weeks in cortex and hippocampus. Thus, it was not surprising that IL-1β levels were elevated in the Tg animals in the striatum at 36 weeks.

IL-1β levels become elevated in regions of high Aβ deposition at 28 weeks and more regions become affected at 36 weeks. CXCL1 initially was significantly elevated in the olfactory bulb of
Tg animals compared to wild-type, with differences in the cortex and hippocampus approaching significance.

Microglia and astrocytes are capable of secreting IL-1β (Vandenabeele et al., 1991). IL-1β stimulation has been shown to increase secreted levels of CXCL1 in HeLa cells (Yang et al., 2008). Similarly, in primary mouse granulosa cells, IL-1α elicited an increase in both mRNA and protein levels of CXCL1 (Son and Roby, 2006). These studies suggest that Aβ causes the secretion of IL-1β and IL-1β might in turn stimulate secretion of CXCL1. The sequential increase in Aβ, then IL-1β and finally CXCL1 is reflected in the TgCRND8 mouse model. Aβ began to deposits at 16 weeks and as Aβ continued to deposit, elevated levels of IL-1β appear in regions where Aβ deposition was greatest. This effect achieves significance by 28 weeks. As elevated IL-1β levels became more widespread, CXCL1 levels became elevated, as observed in olfactory bulb at 36 weeks.

These results support my hypothesis that Aβ deposition elicits region-specific inflammatory responses. Brain regions that deposited the most Aβ, olfactory bulb, cortex and hippocampus were also regions of elevated IL-1β expression at 28 weeks. IL-1β levels in the cortex and hippocampus are significantly higher in the TgCRND8 mice compared to wt littermates at 28 weeks and by 36 weeks, the difference became significant in the olfactory bulb and striatum. At 36 weeks, Aβ deposition in the striatum were comparable to the high depositing regions cortex and hippocampus at 28 weeks so it was not surprising that IL-1β levels become elevated in the striatum at 36 weeks. CXCL1 levels became elevated by 36 weeks, followed the elevation of IL-
CXCL1 levels were significantly elevated in the olfactory bulb and higher in the cortex and hippocampus of Tg animals compared to wt littermates.

This cascade of events may be exacerbated by positive feedback mechanisms. IL-1 is capable of stimulating the activity of APP promoter and up-regulating APP mRNA levels (Goldgaber et al., 1989; Lahiri et al., 1995; Yang et al., 1998). IL-1β has also been shown to stimulate γ-secretase activity to increase Aβ40 production (Liao et al., 2004). In addition, CXCL1 stimulation of its receptor CXCR2 increased Aβ40 production (Bakshi et al., 2008).

Levels of inflammatory mediators are at low background levels or are undetectable in healthy individuals whereas in AD patients, higher levels are observed (Akiyama et al., 2000). It is not certain whether increased levels of inflammatory mediators are beneficial or detrimental to the development of AD. Enhancing IL-1β signaling using IL-1 receptor antagonist knockout mice increased both IL-1β levels and TNF-α levels, which resulted in reduced post-synaptic marker PSD95 expression following Aβ infusion (Craft et al., 2005). In contrast, an IL-1β inducible APP/PS1 mouse model showed a decrease in hippocampal plaque load after over-expressing IL-1β for 4 weeks (Shaftel et al., 2007). A decrease in both insoluble Aβ40 and Aβ42 were observed after the IL-1β over-expression (Shaftel et al., 2007). Studies on elevated levels of TNF-α have also shown both beneficial and detrimental effects on AD. Astrocyte but not neuronal over-expression of TNF-α results in neuronal degeneration and astrogliosis as well as microgliosis (Akassoglou et al., 1997). However, pre-treatment with TNF-α in rat hippocampal culture attenuated Aβ-induced neuronal degeneration (Barger et al., 1995). More specifically,
TNF-α pre-treated cultures confer protection against peroxide oxidative stress and glutamate excitotoxicity (Barger et al., 1995).

Even though inflammatory mediators tend to have dual functions, neuroinflammation in the AD brain correlates better with synaptophysin immunoreactivity than do either Aβ or neurofibrillary tangles (Lue et al., 1996). There are cases of non-demented individuals expressing levels of either Aβ or neurofibrillary tangles found in AD patients at autopsy. These individuals do not exhibit any symptoms of AD (Kutty et al., 1994). Neuroinflammation could be a missing link between Aβ accumulation and disease presentation. In support of the notion that neuroinflammation is deleterious, these non-demented individuals with protein aggregates in their brain had slightly elevated inflammatory markers but much less so than those encountered in AD patients (Lue et al., 1996).
Chapter 6
Conclusion

The temporal sequence Aβ deposition observed in TgCRND8 mice as is reported in AD patients (Thal et al., 2002). Insoluble Aβ accumulation in the TgCRND8 mice started in the olfactory bulb and cortex at 16 weeks of age, then hippocampus at 28 weeks. Accumulation in the striatum and septum is slower and did not catch up to levels in the olfactory bulb, cortex and hippocampus. Levels of insoluble Aβ in each region is reflected by the amount of Aβ plaques observed in that region, with the exception of the cerebellum. No plaques were seen in the cerebellum despite moderate insoluble Aβ levels.

Factors that may contribute to differential regional Aβ accumulation were examined. Regional Aβ analysis showed delayed peak Aβ42/Aβ40 ratio in the striatum and low peak Aβ42/Aβ40 ratio in the septum relative to ratios in other regions. These factors may have contributed to slow accumulation of insoluble Aβ in these two regions. Regional analysis of Aβ species also revealed an increase in Aβ oligomers in the hippocampus, which may contribute to the spatial memory deficit associated with hippocampus.

In response to regional Aβ accumulation, a temporal association between Aβ and inflammatory mediators IL-1β and CXCL1 have also been found. Aβ accumulation starts at 16 weeks and IL-1β levels became elevated in regions with greater Aβ accumulation at 28 weeks. At 36 weeks, elevated levels of CXCL1 were observed in the brain.
Chapter 7
Future Directions

To further decipher the relationship between Aβ accumulation and cytokine production, a correlation analysis was performed to determine a link. As shown in Fig. 20, insoluble Aβ40 levels correlate with IL-1β levels starting at 28 weeks (r = 0.823, p-value = 0.044) (Fig. 20A). This correlation becomes stronger as more Aβ40 deposits (r = 0.976, p-value = 0.0008) (Fig. 20B). On the other hand, Aβ42 levels correlate with CXCL1 levels at 36 weeks when CXCL1 levels start to become elevated in Tg animals (r = 0.865, p-value = 0.026) (Fig. 20C). Future experiments are required to determine why the two types of Aβ stimulate different inflammatory mediators. Immunohistochemical analysis would help to determine the localization of IL-1β and CXCL1 relative to Aβ42 and Aβ40. In vitro cell culture experiments with Aβ42 and Aβ40 stimulation would also help to confirm and explain the Aβ and cytokine associations.

The relationship between Aβ, IL-1β and CXCL1 can also be evaluated in vivo. In TgCRND8 mice, Aβ can be neutralized both prior and post the manifestation of inflammation. To determine the effect of Aβ load on levels of IL-1β and CXCL1, Aβ load can be reduced at 16 weeks before elevated levels of IL-1β occurs to find out if reduced Aβ would reduce inflammation mediated by IL-1β. Aβ can also be reduced at 28 weeks after IL-1β elevation but before CXCL1 elevation to determine if CXCL1 elevation is triggered by IL-1β or Aβ and if reducing Aβ could lessen the effect of CXCL1. Aβ load can be reduced by antibodies and small molecules such as scyllo-inositol. To further study the cause of elevated levels of CXCL1
observed at 36 weeks, whether stimulated by Aβ or IL-1β, TgCRND8 mice can be crossed with IL-1R knockout mice to evaluate the effect of disrupted IL-1β signaling.

Figure 20. Cytokine levels are correlated with insoluble Aβ load. Aβ40 positively correlates with IL-1β at 28 weeks ($r = 0.823, p = 0.044$) and a stronger correlation at 36 weeks ($r = 0.976, p = 0.0008$). Aβ42 correlates with CXCL1 at 36 weeks ($r = 0.865, p = 0.026$).
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