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Amarallys Cintron Date

Cellular trafficking and molecular heterogeneity of Aβ seeds

How similar are β-amyloid aggregates and prions?

By

Amarallys Cintron

Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

Neuroscience

_____

Lary C. Walker, Ph.D.
Advisor

_____ _____

Victor Faundez, M.D., Ph.D
Committee Member

Brian Ciliax, Ph.D.
Committee Member

_____ _____

Thomas Kukar, Ph.D
Committee Member

Harry LeVine, III, Ph.D.
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____

Date

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Amarallys Cintron

B.Sc. University of Florida, 2007

Advisor: Lary C. Walker, PhD.

An abstract of
A dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University
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Abstract

Cellular trafficking and molecular heterogeneity of Aβ seeds

How similar are β-amyloid aggregates and prions?

By Amarallys Cintron

As the deposition of aggregated Aβ peptide in the brain parenchyma is an obligatory event in Alzheimer's disease, Aβ has become the subject intensive study. In this thesis work, my primary aim has been to investigate key similarities between Aβ seeds and prions, the proteinaceous agents that underlie spongiform encephalopathies. Research on prion diseases established the novel concept that certain proteins can cause disease by a process involving misfolding and aggregation. As such, the prion paradigm has increasingly found parallels in more common neurodegenerative diseases. I have investigated several prion-like characteristics of Aβ seeds, including the durability of the seeds, transport of seeds by immune cells, and the existence of structural/functional variants (strains) of the seeds. I found that, like prions, Aβ seeds are resistant to destruction by formaldehyde fixation. Specifically, fixation does not eliminate the ability of Aβ seeds to induce the formation of plaques and cerebral Aβ angiopathy in an Aβ precursor protein- (APP) transgenic mouse model of cerebral Aβ deposition. Inflammation commonly occurs in numerous neurodegenerative diseases. In Alzheimer's disease, some studies have shown that macrophages are beneficial to plaque clearance, but others suggest that plaques contribute to the deposition of Aβ. Here, I demonstrate that macrophages can take up Aβ seeds in the periphery and, in small numbers, transport them across the blood brain barrier in mice. While this finding indicates that macrophages could contribute to the spread of the seeds, a preliminary investigation of the long-term effects of exogenous macrophages bearing human, Aβ-rich brain extract yielded a small decrease in brain Aβ in APP-transgenic mice seven months later. This paradoxical effect suggests the activation of an adaptive immune response by seed-bearing cells, a possibility that warrants further investigation. Lastly, I undertook an analysis of prion-like strain differences in Aβ. The imaging compound Pittsburgh Compound B (PIB) binds with high specificity and affinity to Aβ in human brains. By seeding APP-transgenic mice with Aβ-rich brain extracts, I was able to induce the deposition of Aβ, which stimulates PIB binding to the deposits at an early age. Because these PIB-positive deposits are induced in the mice by human, nonhuman primate and murine Aβ seeds, it appears that seeding acts to accelerate the maturation of the lesions, and that the molecular conformation of Aβ deposits can evolve with age. Taken together, these experiments support the concept that Aβ seeds share important structural and biological features with prions.

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Chapter 1:

Introduction and Background

1.1 A brief history of Alzheimer's disease

In 1901, Alois Alzheimer, a German neuropathologist and psychiatrist, encountered a 51 year old patient named Auguste Deter whose symptoms included impaired memory, aphasia, disorientation, and psychosocial incompetence (Alzheimer, 1907; (Alzheimer et al., 1995). Her condition steadily worsened, and she was diagnosed with what came to be called *presenile dementia*. Deter died four and a half years later, and using recently developed methods for examining tissue sections under the microscope, Alzheimer examined her brain for clues that might explain her condition. He attributed her dementia to "miliary foci" and neurofibrillary change in the cerebral cortex. The miliary foci were extracellular parenchymal ('senile') plaques, and the neurofibrillary lesions were intracellular neurofibrillary tangles. Alzheimer first described these findings at a meeting in Tübingen, Germany in 1906, and published them in 1907 (Alzheimer, 1907). In 1911, after several additional cases had been discovered, the disease was formally given the name 'Alzheimer's Disease' by Kraepelin (Maurer et al., 1997).

In the 100+ years since the first case report was described, much more has been learned about Alzheimer's disease. The pace of research was dramatically accelerated by the identification in the 1980's of the specific proteins that form senile plaques and neurofibrillary tangles – amyloid-beta (Aβ) and tau, respectively. Knowledge of their amino acid sequences soon led to the production of highly specific antibodies to these proteins, and to the discovery of genetic mutations that cause Alzheimer's disease as well as related disorders known as the primary tauopathies (Chartier-Harlin et al., 1991; Forman et al., 2004; Goate et al., 1991; Hong et al., 1998; Mullan et al., 1992; Trojanowski and Mattson, 2003).

1.2 Dementia and Alzheimer's disease

Dementia is broadly defined as a decline in mental ability severe enough to interfere with daily life. Although there are numerous causes of dementia, Alzheimer's disease accounts for 60-70% of all neurodegenerative dementia cases (Barker et al., 2002). The two most prevalent risk factors for dementia are advancing age and genetics. The incidence of Alzheimer's disease doubles every 5 years after the age of 65 (Alzheimer'sAssociation, 2012; Hendrie, 1998; Kawas and Corrada, 2006). The other major risk factor, genetics, is two-fold: deterministic genes and risk genes. Those who have deterministic (autosomal dominant) genetic mutations, if they live long enough, will consistently develop Alzheimer's disease, whereas those who possess 'risk genes' have an increased likelihood of being diagnosed with Alzheimer's disease, but the eventual emergence of the disease in those carrying the specific genetic polymorphisms is not a certainty. Studies have identified mutations in deterministic genes that code for the proteins amyloid-β (Aβ) precursor protein (APP), presenilin-1, and presenilin-2. The most prevalent risk factor gene codes for a particular variant of a lipoprotein involved in lipid transport and metabolism known as apolipoprotein E4 (ApoE4) (Yu et al., 2014). The APOE gene in humans exists as three different alleles, termed APOE-2, APOE-3 and APOE-4. The protein isoforms encoded by these alleles appear to differentially influence the risk of developing Alzheimer's disease. Persons bearing either APOE-3 or APOE-2 are not at increased risk of developing Alzheimer's disease, whereas the risk increases in a gene dose-dependent manner in those expressing one or two APOE4 alleles (Rebeck et al., 2002).

Alzheimer's is fundamentally a disease of aging. As a result of improvements in lifestyle and advances in healthcare, more people are living longer, thus increasing the incidence of

Alzheimer's disease. Currently, more than 5 million people are living with Alzheimer's disease in the United States, and it is estimated to be the 6th leading cause of death (Alzheimer'sAssociation, 2012). In fact, Alzheimer's is very likely an even more frequent cause of death, as the reporting of dementia on death certificates greatly underestimates the actual prevalence of the disorder in the elderly (Wachterman et al., 2008). Alzheimer's disease thus is responsible for an enormous strain on healthcare, with a direct cost in the U.S. of $214 billion in 2013 (Alzheimer'sAssociation, 2012). The prevalence of Alzheimer's disease is expected to triple by 2050 as the 'baby boom' generation ages. Importantly, Alzheimer's disease is the only disease in the top 10 fatal diseases in America that cannot be effectively treated (Alzheimer'sAssociation, 2012), and there is no therapy that can either slow or prevent this devastating disorder.

Current diagnostic procedures for Alzheimer's disease include a neurological and physical assessment, medical history, and a mental health test. An important objective of the examination is to rule out other potential causes of dementia, some of which may be treatable. There are a number of tests to assess dementia, one being the Clinical Dementia Rating scale (CDR), a useful tool to determine the severity of an individual's dementia (Morris, 1993). Those who develop very mild cognitive deficits meet the criteria for a diagnosis of Mild Cognitive Impairment (MCI). Many patients diagnosed with MCI later are diagnosed with Alzheimer's disease, although incipient AD is not the only cause of MCI (Mitchell and Shiri-Feshki, 2009).

Advancing technology has introduced new diagnostic tools for detecting amyloid plaques and neurofibrillary tangles in a living patient's brain. Several imaging agents have been developed to

detect Aβ deposits, notably Pittsburgh Compound B (PIB), a brain-penetrant, radiolabeled benzothiazole derived from the amyloid-staining dye Thioflavin-T (Biancalana and Koide, 2010; Klunk et al., 2001). PET scanning using ^{11}C-PIB, which crosses the blood-brain barrier and binds to amyloid plaques and cerebral amyloid angiopathy (see below), enables the visualization of amyloid load in the brains of living patients (Clark et al., 2011; Klunk et al., 2004). These techniques have only emerged within the past few years, but they are proving to be valuable tools in the diagnosis of Alzheimer's disease, as well as in determining whether the cognitive impairment in a patient with MCI is likely to be due to an Alzheimer-like pathogenic process. Recently, promising compounds have been introduced that selectively detect tauopathy in the living brain (Fodero-Tavoletti et al., 2011; Maruyama et al., 2013; Zhang et al., 2012), though their development remains behind that of Aβ-imaging agents (Villemagne and Okamura, 2014).

1.3 The neuropathology of Alzheimer's disease

1.3.1 Amyloid and Aβ (β-amyloid). *Amyloid* is a generic term that is used to describe abnormal accumulations of misfolded proteins in various cells and tissues of the body. In the 1800's, 'amyloid' was coined to describe the starch-like staining pattern of certain lesions, but eventually it was determined that the stained material consists mainly of protein (Sipe and Cohen, 2000). The term *amyloid* persisted, however, as an adjective that eventually became the noun that we use today to describe proteinaceous lesions with the following characteristics: birefringence when viewed through cross-polarizing filters after staining with the dye Congo Red; long, unbranched fibrils of ~10nm diameter under the electron microscope; and a cross-β quaternary structure by X-ray fiber diffraction analysis (Jucker and Walker, 2013; Westermark et al., 2005). Interestingly, amyloid can be formed by many proteins with quite different amino acid sequences

(Westermark et al., 2005), and indeed, under the right conditions, all proteins have the potential to form amyloid (Dobson, 1999). The main form of amyloidosis in the brain consists of fibrillar masses of the Aβ peptide (Glenner and Wong, 1984; Glenner et al., 1984; Hardy and Selkoe, 2002; Holtzman et al., 2011; Masters et al., 1985), a cleavage product of APP that, in its soluble form, is normally generated in small amounts in the brain (Busciglio et al., 1993; Haass et al., 1992; Hardy and Selkoe, 2002; Seubert et al., 1992; Shoji et al., 1992).

One of the earliest clues that led to the identification of Aβ came from Down's syndrome (trisomy 21). Patients with Down's syndrome have been known for many years to develop Alzheimer's disease early in life (Holland et al., 2000; Mann, 1988a; Mann, 1988b), often in their 30's or 40's, but the cause of this premature neurodegenerative change was unknown. Studies analyzing the molecular pathology of Alzheimer's disease and Down's syndrome yielded an unexpected but important finding; determination of the amino acid sequence of Aβ enabled scientists to localize the gene that codes for Aβ and its precursor, APP, to chromosome 21 (Korenberg et al., 1989). This finding provided some of the first evidence that Aβ might be a trigger for Alzheimer's disease, in that the extra copy of the APP gene in trisomy 21 results in the overproduction of the precursor and, by extension, Aβ - the primary component of senile plaques in Alzheimer's disease (Golde et al., 2000; Hardy and Selkoe, 2002).

To liberate Aβ, the membrane-spanning precursor APP is sequentially cleaved by two membrane-bound endoproteases, β-secretase (also known as BACE) and gamma secretase (γ-secretase) (Haass, 2004). Another cleaving enzyme, alpha-secretase (α-secretase), cuts Aβ in two, thereby preventing it from forming aggregates (De Strooper and Annaert, 2000; Haass,

2004; Murphy and LeVine, 2010). Aβ can exist in varying lengths, most often consisting of a string of 40 or 42 amino acids. The peptide can be further truncated at both the C- and N-termini, yielding a large variety of fragments of varying sizes (De Strooper and Annaert, 2000; Masters et al., 1985; Miller et al., 1993; Murphy and LeVine, 2010; Prelli et al., 1988; Selkoe, 2001). In addition, Aβ can be post-translationally modified, such as by the pyroglutamization of amino acids 3 or 11 in fragments that have been truncated at these residues (Mori et al., 1992; Perez-Garmendia and Gevorkian, 2013). The first genetic (autosomal dominant) mutations that were discovered to cause AD were found within the APP gene (Goate et al., 1991). Subsequently, an even larger number of mutations were found in the genes that encode the presenilins, proteins that are essential components of the intramembranous APP-cleaving enzyme gamma-secretase (Hardy and Selkoe, 2002; Sherrington et al., 1995). Later, mutations in the gene for the tau protein were determined to cause primary tauopathies such as frontotemporal dementia, but it is informative from the standpoint of pathogenesis that tau mutations do not cause the senile plaques that are characteristic of AD (Gotz et al., 2004) (see below).

These findings provided compelling evidence for the hypothesis that the accumulation of Aβ is the initial cause of Alzheimer's disease (Hardy and Selkoe, 2002). The autosomal dominant Alzheimer's disease mutations either: 1) cause more Aβ to be produced, or 2) result in a species of Aβ that is more prone to aggregate and form fibrils, particularly Aβ1-42. The fibrillar form of Aβ constitutes the cores of the senile plaques that are prominent in Alzheimer's disease. However, it is important to note that there are smaller, soluble aggregates of Aβ that can be particularly toxic to cells, and therefore may be the most pathogenic manifestation of the

aggregated peptide (Catalano et al., 2006; Gong et al., 2003; Goure et al., 2014; Haass and Selkoe, 2007; Klein, 2013; Lesne et al., 2006; Lesne, 2014; Walsh et al., 2002). In either case, current evidence indicates that the misfolding and abnormal aggregation of Aβ is a key, early occurrence in the pathogenesis of AD, a concept that has been encapsulated by the 'amyloid cascade hypothesis' of AD (Hardy, 2002).

1.3.2 Tau protein. According to the amyloid cascade hypothesis, the initial misfolding and multimerization of Aβ drives a series of subsequent changes, one being the ectopic polymerization of the principal component of neurofibrillary tangles, tau protein (Blurton-Jones and Laferla, 2006; Oddo et al., 2006). Tau is a microtubule-associated protein that, when hyperphosphorylated, becomes insoluble and forms filamentous inclusions in cells. The filaments constitute the intracellular neurofibrillary (tau) tangles found in Alzheimer's disease. Alzheimer's disease is one of many diseases that are referred to as *tauopathies* (Kovacs, 2015; Lee et al., 2001; Spillantini and Goedert, 2013). These disorders can result from mutations in the gene that encodes the tau protein, or they are secondary to such insults as head injury (chronic traumatic encephalopathy) or abnormalities in other proteins such as Aβ. The tauopathies thus are clinically diverse disorders. In AD, although the evidence strongly indicates that Aβ aggregation is upstream of neurofibrillary tangles in the pathogenic cascade, the tauopathy nonetheless is a critical contributor to dementia (Braak and Braak, 1991; Crystal et al., 1988; Jucker and Walker, 2011; Jucker and Walker, 2013; Walker and LeVine, 2012; Wilcock and Esiri, 1982). Furthermore, alterations in tau have not been shown to induce the amyloid plaques that are characteristic of AD, indicating that the amyloid cascade is unidirectional with regard to the two cardinal lesions of the disease. This unidirectionality is supported by studies of

transgenic mouse models expressing both APP and tau (Gotz et al., 2001; Gotz et al., 2004; Howlett and Richardson, 2009).

1.4 Molecular mechanisms of protein aggregation: The prion paradigm

The functionality of a protein is highly dependent upon its 3D structure. Although entire systems are designed to ensure that a protein folds correctly after translation, many diseases are associated with protein misfolding events. These misfolding events result in a loss of protein function or a gain of toxic function, and often are accompanied by the accumulation of the protein as extracellular deposits and/or intracellular inclusions. In many protein misfolding diseases, the protein misfolds and converts normally soluble forms into insoluble multimers, particularly amyloid (Dobson, 1999). Collectively, diseases involving the misfolding and abnormal accumulation of certain proteins are referred to as 'proteopathies' (Walker and LeVine, 2000).

The prion diseases are a special type of proteopathy that exhibit the unusual property of being heritable, sporadic (idiopathic) or infectious in origin (Brown et al., 1982; Gajdusek, 1994; Prusiner, 1984). The term prion was coined to refer to 'proteinaceous infectious particles', referring to the disease-causing agent in the spongiform encephalopathies. In humans, prion diseases are rare; they include Creutzfeldt-Jakob disease, fatal insomnia, Gerstmann-Sträussler-Scheinker syndrome, and kuru (McKintosh et al., 2003; Prusiner and Hsiao, 1994; Prusiner, 2013). There are also a number of prion diseases of nonhuman species, such as scrapie in sheep and bovine spongiform encephalopathy in cows (Hope et al., 1988; Imran and Mahmood, 2011; Mathiason et al., 2006; Prusiner, 1998). In the prion diseases, a misconformed, β-sheet rich

prion protein acts as a template for the normal (pathogenically benign) prion protein, directing the benign form of the protein to itself misfold and aggregate. Though the precise mechanism of toxicity is not entirely clear, in this self-aggregated state, these malformed prion proteins are injurious to neurons (Prusiner, 2001). The prion diseases are the only known directly infectious cerebral proteopathy; in other words, they are able to pass from one organism to another and cause disease in the recipient. Other cerebral proteopathies are not infectious in this sense. However, there is considerable evidence to indicate that a prion-like molecular mechanism is involved in the origination and progression of many neurological and systemic diseases (Aguzzi and Rajendran, 2009; Brundin et al., 2010; Chu and Kordower, 2015; Frost and Diamond, 2010; Goedert et al., 2010; Morales et al., 2015; Soto et al., 2006; Walker et al., 2006; Walker and Jucker, 2015; Westermark and Westermark, 2010).

Alzheimer's disease shares a number of similarities with prion diseases pathogenically, and as such has been suspected of being inducible in a prion-like manner (Prusiner, 1984). A transgenic mouse paradigm pioneered by the Walker and Jucker laboratories has demonstrated that intracerebral injections of brain extracts that contain large amounts of multimeric Aβ (Aβ seeds) stimulate the premature formation AD-like Aβ lesions (Kane et al., 2000; Rosen et al., 2012). Injection of Aβ-depleted or neutralized extracts produces no effect (Meyer-Luehmann et al., 2006). In addition, injection of aggregates of pure, synthetic Aβ also stimulates plaque formation in transgenic mouse models (Stohr et al., 2012), thereby demonstrating that aggregated Aβ is the necessary element for inducing the pathology (Meyer-Luehmann et al., 2006). Indirect evidence indicates that the seeds travel via axonal transport, as injection into one brain region triggers deposits in axonally connected regions (Jucker and Walker, 2011; Ye et al., 2015).

Furthermore, the introduction of Aβ seeds into the peritoneal cavity can induce Aβ deposition in the brain (Eisele et al., 2010; Eisele et al., 2014). The prevalence of amyloid angiopathy in the forebrain of intraperitoneally seeded APP23 transgenic mice (Eisele et al., 2010) suggests the possibility that the seeds reach the brain via the vasculature (Eisele et al., 2014). Furthermore, the presence of Aβ within circulating macrophages of these mice implicates these cells as possible vectors for the transport of seeds from periphery to brain (Eisele et al., 2014). Aβ seeds also demonstrate evidence of prion-like structural and functional variations, often referred to as proteopathic *strains* (Walker and Jucker, 2015), the properties of which can be faithfully transmitted to susceptible mouse models (Fritschi et al., 2014; Heilbronner et al., 2013; Stohr et al., 2014; Watts et al., 2014). Prion strains arise from alternative stable conformations of prion protein that can be reliably propagated (Morales et al., 2007).

The prion-like misfolding and seeded aggregation of proteins is not confined to Aβ. The tau protein also has been shown to have prion-like properties *in vivo* (Clavaguera et al., 2009; Clavaguera et al., 2013; Clavaguera et al., 2014; Falcon et al., 2015; Holmes et al., 2014; Iba et al., 2013; Lasagna-Reeves et al., 2012; Peeraer et al., 2015; Stancu et al., 2015; Tolnay and Probst, 1999); the intracerebral injection of aggregated tau induces tauopathy in tau-transgenic mice, and these lesions are propagated among axonally connected brain regions (Clavaguera et al., 2009; Clavaguera et al., 2015). In addition, the focal expression of transgenic tau protein in the mouse brain induces tauopathy that systematically spreads to interconnected sites, suggestive of the axonal transport of a pathogenic agent (Hyman, 2014; Liu et al., 2012; Walker et al., 2013).

There is now little doubt that protein misfolding and aggregation are crucial events in many diseases, especially Alzheimer's disease. Much insight can be gained into Alzheimer's and other proteopathies by investigating the self-assembly, transport, and spread of these seeds in the misfolded protein propagation model. Knowing the conditions under which proteins misfold and how they begin templating benign proteins could eventually yield therapeutic interventions that can prevent the domino effect seen in these proteopathies. The impact of investigation into protein misfolding and propagation goes far beyond Alzheimer's disease. In recent years, the list of proteins that appear to emerge, proliferate and cause disease by a prion-like process has grown dramatically, and includes Aβ, α-synuclein, TDP-43 and many others (Aguzzi and Rajendran, 2009; Braak et al., 2003; Goedert et al., 2014; Grad et al., 2015; Jucker and Walker, 2011; Munch and Bertolotti, 2010; Polymenidou and Cleveland, 2012; Walker and Jucker, 2015; Westermark and Westermark, 2010). Evidence for prion-like inducibility even has been found in some cancers, cystic fibrosis, and systemic amyloidoses (Dobson, 1999; Silva et al., 2014; Westermark and Westermark, 2010). How does the prion-like cascade of protein aggregation begin? In AD, growing evidence implicates inflammation as an important feature that is closely involved in the disease process.

1.5 Inflammation and AD

The immune system is the body's defense against foreign elements. The immune response is tightly controlled and heavily regulated, which is especially evident as it relates to the limited access of systemic immune mediators to the central nervous system (CNS) (Wyss-Coray and Mucke, 2002). Stroke, trauma, infections, and other disorders all cause upregulation of the immune response in the brain, which normally consists of the activation of endogenous glial

cells, in particular microglia and astrocytes, and to the constrained trafficking of systemic immune cells such as monocytes into the brain (Akiyama et al., 2000; Rogers et al., 1988; Togo et al., 2002). Other cell types implicated in the brain immune response are blood vessel-associated pericytes and perivascular macrophages (Guillemin and Brew, 2004; Hill et al., 2014; Perry, 2004; Rivest, 2009). The origins and functions of these perivascular cells are incompletely understood, but their location at the interface of the blood and brain positions them to mediate immune cross-talk between the periphery and the CNS (Guillemin and Brew, 2004; Lampron et al., 2013; Perry, 2004).

Immune function can be broadly classified into two categories: the innate immune response and the adaptive immune response. The *innate* immune response is responsible for rapidly recruiting cells to sites of infection using cytokines as chemical mediators. The innate response mediates the identification of foreign substances and microbes, followed by the activation of cells specialized for removal of the invaders. Clearance is typically facilitated by various types of macrophage in the periphery, and in the CNS by the resident macrophages, the microglia. The *adaptive* immune response involves the presentation of antigens by specialized ('antigen-presenting') cells and the generation of antibodies that specifically target antigens on the invasive agent, thereby further stimulating a cytological response and thus maximizing the body's ability to clear threats (Alberts et al., 2002). When a pathogen is encountered and recognized as foreign, it is usually phagocytosed by a macrophage (the most effective type of phagocyte), neutrophil, or dendritic cell. The phagocyte processes the pathogen and displays pathogen-derived antigens on its cell surface. A T-helper cell interacts with the phagocyte, which then releases interleukin-1, thereby stimulating the T-helper cell to release interleukin-2.

Interleukin-2 stimulates the division of B and T cells, and also recruits various cytotoxic T cells that destroy infected cells (Parkin and Cohen, 2001). The adaptive immune response also is responsible for the production of memory cells, which constitute an enduring database of effective B and T lymphocytes. As a result, when the antigen is encountered again, the secondary (adaptive) immune response will be swifter and stronger (Alberts et al., 2002; Janeway et al., 2001).

In the CNS, the innate immune response results in the activation of the brain's principal resident immune cells, microglia, which, in their activated state, adopt a phagocytic phenotype (Kettenmann, 2013). This change is followed by the release of inflammatory mediators: cytokines and chemokines. The neuroinflammatory response is typically short-lived, and like inflammation elsewhere in the body is geared toward minimizing damage to cells and tissues by neutralizing destructive threats. However, chronic inflammation also can be detrimental to surrounding neurons, as the levels of cytokines and chemokines remain high and the microglia remain active (Perry et al., 2010). This chronic response results in oxidative stress, much to the detriment of local cells (Tansey et al., 2007). Neurodegenerative diseases, including Alzheimer's disease, are marked by chronic inflammation and elevated levels of cytokines (Block and Hong, 2005; Heneka et al., 2015; Mrak and Griffin, 2005). Often, signs of neuroinflammation are present before extensive neurodegeneration is evident (Frank-Cannon et al., 2009). Thus, it is important to understand the role of inflammation and the immune response as they relate to the disease course in Alzheimer's disease.

1.6 Cellular mechanisms in AD

As described above, a chronic inflammatory state is injurious to surrounding neurons. In AD, a pathogenic trigger (or triggers) is responsible for impairing neuronal function, and the aberrant accumulation of Aβ plays a role in this process. The details of this sequence of events remain only partially understood, but it is clear that microglia and astrocytes become activated as a part of the innate immune response in the AD brain. Cytokines and other chemical signals expressed by glial cells and dying neurons sustain a vicious cycle in which microglia and astrocytes are further activated. The feedback loops among neurons and glial cells create a prolonged release of neuroinflammatory markers, causing cellular damage and exacerbating neurodegeneration (Wyss-Coray and Mucke, 2002). Perivascular cells also contribute to the pathologic phenotype in AD. For instance, aggregates of Aβ are found within degenerating pericytes (Verbeek et al., 1997; Wilhelmus et al., 2007), and the dysfunction of pericytes correlates with a weakening of the BBB, which could in turn secondarily promote neuronal degeneration (Daneman et al., 2010).

The role of microglia in the clearance of Aβ is controversial. A study in which microglia were selectively ablated in a mouse model detected no difference in Aβ plaque load, suggesting a limited role of microglia in Aβ clearance (Grathwohl et al., 2009). Another study showed that an APP-transgenic mouse that was crossed with a mouse overexpressing factors that enhanced microgliosis resulted in facilitated Aβ uptake and intracellular Aβ oligomerization (Kiyota et al., 2009). Evidence has suggested that peripheral (blood-derived) macrophages may be more adept at Aβ clearance than are microglia (Frackowiak et al., 1992; Wisniewski et al., 1989; Wisniewski et al., 1991). Microglia by definition are restricted to the CNS, where they have long lifespans

and a limited ability to proliferate (Ajami et al., 2007; Gate et al., 2010). Peripheral macrophages are much more dynamic, and are known to be recruited to the brain following injury (Gate et al., 2010). Furthermore, populations of macrophages are capable of migration into the brain parenchyma, where they assume a microglial phenotype (Priller et al., 2001). Studies that induced a weakening of the BBB report an increased infiltration of peripheral macrophages into the brain (Mildner et al., 2007). This process could imitate some of the characteristics of Alzheimer's disease; pericyte degeneration could allow increased numbers of peripheral macrophages to cross the BBB (Lynch, 2014). The precise influence of macrophages on plaque load requires additional investigation. However, it is worth noting that, both in experimental mice and in humans that are immunized against Aβ as a potential treatment for AD, a reduction in plaque load correlates with an increase in the number of Aβ-immunoreactive macrophages (Boche et al., 2010; Masliah et al., 2005; Morgan, 2011; Schenk et al., 1999).

1.7 Animal models of Alzheimer-type neuropathology

Genetically modified mouse models have greatly expanded the scope of experimental and therapeutic investigations of AD-like pathology (Gotz and Ittner, 2008; Howlett and Richardson, 2009; Jucker, 2010). The expression of mutated human APP in the mouse genome results in Aβ plaque deposition similar in many ways to that seen in Alzheimer's disease (Duyckaerts et al., 2008). Numerous mouse models of AD-like Aβ deposition have been generated; however, it is important to emphasize that, although these models simulate some pathology of AD, they do not develop all features of AD (Jucker, 2010). In fact, even aged nonhuman primates, which naturally manifest abundant senile plaques and cerebral amyloid angiopathy in old age, do not show other signs of AD (such as neurofibrillary tangles, brain shrinkage, and profound cognitive

loss) (Heuer et al., 2012). This caveat notwithstanding, these animal models have proven to be useful tools in understanding cellular and molecular mechanisms of AD pathogenesis, including the production, fibrillization and transport of Aβ. Furthermore, use of these animal models has led to the discovery of new therapeutic targets and to numerous experimental trials for Alzheimer's disease treatments.

1.8 Therapeutic approaches to AD

The treatments for Alzheimer's disease that are currently approved for use in the U.S. are only of limited efficacy in ameliorating the signs and symptoms of the disease (Walker and Rosen, 2006). There are no existing therapies that prevent or slow disease progression. Acetylcholinesterase inhibitors (AChEI) are the most commonly prescribed type of therapy. These drugs emerged from the discovery that cholinergic neurons are profoundly depleted in end-stage AD (Bartus, 2000; Perry, 1980). The neurotransmitter acetylcholine had previously been implicated in memory function (Bartus et al., 1982; Bartus et al., 1985), and studies in the 1970's showed a 30-50% reduction of its synthetic enzyme choline acetyltransferase in AD brains compared to controls (Bowen and Davison, 1980). Acetylcholinesterase inhibitors increase the amount of acetylcholine in synapses by preventing acetylcholinesterase from breaking down the neurotransmitter. Though the AChEIs have been shown to improve the symptoms in some patients, the benefits are transient, and the disease maintains its ineluctable downhill course (Farlow, 2002; Galimberti and Scarpini, 2011; Walker and Rosen, 2006).

Another neurotransmitter system that is affected in Alzheimer's disease is the glutamatergic system. Glutamate, the main excitatory neurotransmitter in the vertebrate brain (Meldrum,

2000), is thought to be involved in learning and memory via the establishment of long-term potentiation (Francis et al., 1993). Hyperactivity of the glutamatergic system leads to the excessive influx of calcium ions in recipient neurons, which causes them to degenerate by an excitotoxic mechanism (Molino et al., 2013). In this way, hyperactivity of the glutamatergic system has been hypothesized to damage neurons in Alzheimer's disease. Memantine is an NMDA receptor antagonist that binds to NMDA receptors, thereby protecting them from glutamate overactivation (Grossberg et al., 2009). Memantine (Namenda) is prescribed to ameliorate some functional deficits in late-stage AD (Lleo et al., 2006; Marder, 2004), but, like the cholinesterase inhibitors, the drug does not slow the progression of the disease (Yiannopoulou and Papageorgiou, 2013). The inability of drugs targeted to individual transmitter systems to modify the disease course in AD is probably due to the fact that many types of neurons are devastated in AD (Wilcock et al., 2007). More effective therapies thus should be directed toward upstream, common instigators of neurodegeneration, a prominent candidate being the aggregation of Aβ (Walker and Rosen, 2006).

A variety of therapeutic approaches to AD that are based on the amyloid cascade hypothesis are currently under development (Yiannopoulou and Papageorgiou, 2013). In theory, such strategies could either lower the production of Aβ, block its aggregation, prevent it from damaging cells, or promote its removal (Cummings, 2008; Gilman et al., 2005; Hock and Nitsch, 2005; Imbimbo and Giardina, 2011; Walker and Rosen, 2006). The first clinical trials designed to test immunization-based therapies using anti-Aβ antibodies, or to lower Aβ production by inhibiting secretases, were not successful (Savonenko et al., 2012; Wisniewski and Konietzko, 2008), even when there was evidence that the Aβ burden in the brain has been lowered (Gilman et al., 2005).

These findings were disappointing, particularly in light of pre-clinical experiments that have shown that both immunization and secretase inhibition can significantly reduce senile plaque burden in transgenic mouse models (Cummings, 2008; Hara et al., 2011; Qu et al., 2010; Rakover et al., 2007; Savonenko et al., 2012).

The clinical trial results thus have challenged the amyloid cascade hypothesis (Selkoe, 2011). However, with the emergence of amyloid imaging agents such as Pittsburgh compound B (PIB), as well as increasingly sensitive assays for disease biomarkers in CSF, it is now apparent that the deposition of Aβ and tau in the brain begins decades before the onset of symptoms (Bateman et al., 2012; Jack et al., 2013; Sperling et al., 2011). In fact, the Aβ plaque load in the brain has peaked by the time the symptoms of dementia appear. Clearly there is a need to understand the earliest events in the pathogenesis of AD, as well as the cellular and molecular mechanisms that promote the onset and progression of the disease.

1.9 Gaps in Knowledge

Research thus far has explored many of the characteristics of Aβ as it relates to Alzheimer's disease. We know that many familial forms of Alzheimer's disease are caused by mutations in APP that promote the misfolding and aggregation of Aβ (Goate et al., 1991; Hardy and Selkoe, 2002). This mechanism is reminiscent of the prion paradigm, which presents a new and compelling explanation for the special pathogenic mechanisms underlying Alzheimer's disease and many other disorders (Jucker and Walker, 2013; Prusiner, 2013; Walker and Jucker, 2015). Several studies have begun to investigate some of the features that prions and Aβ seeds have in

common. They are both protein-only agents that propagate in susceptible hosts by compelling like molecules to adopt a similar, abnormal shape (Jucker and Walker, 2013; Walker and Jucker, 2015; Prusiner, 2013); both agents exist in a range of sizes (Langer et al., 2011; Silveira et al., 2005), they are resistant to boiling (Meyer-Luehmann et al., 2006), and they are variably sensitive to destruction by proteinase K (Langer et al., 2011). Like prions, Aβ can spread to the brain from the periphery (Eisele et al., 2010), and from region to region within the brain (Hamaguchi et al., 2012; Ye et al., 2015). However, it is still unknown whether Aβ seeds resemble prions in their resistance to formaldehyde fixation (Gordon, 1946), the degree to which they form strain-like variants *in vivo* (Heilbronner et al., 2013; Meyer-Luehmann et al., 2006; Rosen et al., 2010; Rosen et al., 2011; Stohr et al., 2014; Watts et al., 2014), and the potential role of immune cells in transporting the seeds from one location to another. This thesis work aims to investigate these questions under the overarching hypothesis that Aβ shares with prions virtually all of the molecular and cellular characteristics that define a newly identified class of pathogenic agents. More specifically, I have investigated the resistance of Aβ seeds to inactivation by formaldehyde, the ability of immune cells to transport Aβ seeds, and the existence of strain-like variants of Aβ in mouse models as defined by the binding of a selective ligand to aggregated Aβ. Understanding these similarities will inform the general applicability of the protein misfolding model, thereby enhancing our understanding of numerous diseases and the development of more effective therapeutics.

Chapter 2:

Aβ seeds resist inactivation by formaldehyde[1]

[1]This chapter is reproduced with minor changes from the original publication: Fritschi SK, Cintron A, et al., Acta Neuropathologica 2014 Oct;128(4):477-84.

2.1 Abstract

Cerebral β-amyloidosis can be exogenously induced by the intracerebral injection of brain extracts containing aggregated β-amyloid (Aβ) into young, pre-depositing Aβ precursor protein-(APP) transgenic mice. Previous work has shown that the induction involves a prion-like seeding mechanism in which the seeding agent is aggregated Aβ itself. Here we report that the β-amyloid-inducing activity of Alzheimer´s disease (AD) brain tissue or aged APP-transgenic mouse brain tissue is preserved, albeit with reduced efficacy, after formaldehyde fixation. Moreover, spectral analysis with amyloid conformation-sensitive luminescent conjugated oligothiophene dyes reveals that the strain-like properties of aggregated Aβ are maintained in fixed tissues. The resistance of Aβ seeds to inactivation and structural modification by formaldehyde underscores their remarkable durability, which in turn may contribute to their persistence and spread within the body. The present findings can be exploited to establish the relationship between the molecular structure of Aβ aggregates and the variable clinical features and disease progression of AD even in archived, formalin-fixed autopsy material.

2.2 Introduction

The deposition of aggregated Aβ peptide in the brain parenchyma is an early and obligatory event in the pathogenesis of Alzheimer's disease (AD) (Hardy and Selkoe, 2002; Holtzman et al., 2012). Studies in several laboratories have demonstrated that Aβ aggregation can be instigated in the living brain by exogenous, Aβ-rich brain extracts (Eisele et al., 2009; Eisele et al., 2010; Hamaguchi et al., 2012; Kane et al., 2000; Langer et al., 2011; Meyer-Luehmann et al., 2006; Morales et al., 2011; Rosen et al., 2012; Stohr et al., 2012; Walker et al., 2002; Watts et al., 2011), and that the causative agent is an aggregated form of Aβ itself (Jucker and Walker, 2013; Meyer-Luehmann et al., 2006; Stohr et al., 2012). These Aβ seeds bear many similarities to classical prions, i.e., aberrant assemblies of misfolded prion protein (PrP) that infect by inducing other PrP molecules to misfold, aggregate, and self-propagate (Jucker and Walker, 2013; Prusiner, 1998).

A remarkable attribute of prions is their resistance to inactivation by formaldehyde, a fixative that has been used for decades to neutralize viruses in the preparation of vaccines (Delrue et al., 2012). In the 1930s, W.S. Gordon discovered that a concentration of formaldehyde that inactivates the virus causing louping ill in sheep fails to disable the agent causing scrapie (now known to be a prion) (Gordon, 1946). The extreme resistance of the scrapie agent to formaldehyde, which has been confirmed in many laboratories, was one of the earliest indications that the pathogenic agent is unorthodox (Pattison, 1965; Pattison, 1972), helping to impel the development of the prion concept (Prusiner, 1998).

Given the theoretical significance of the prion paradigm for understanding a diversity of diseases (Jucker and Walker, 2013; Prusiner, 2013), we asked whether Aβ seeds resemble prions in their resistance to inactivation by formaldehyde. Our findings show that Aβ-rich brain extracts retain

their ability to induce Aβ deposition in APP-transgenic host mice even after the tissue has spent years in formaldehyde fixative. Using luminescent conjugated oligothiophenes (LCOs), our results further indicate that formaldehyde fixation partially modifies plaque morphology but at the same time maintains the molecular architectures that define Aβ strains.

2.3 Materials and methods

Mice

3-4 month-old male and female APP23 transgenic (tg) mice were used as the seed hosts for all studies(Sturchler-Pierrat et al., 1997). The mice had been backcrossed with C57BL/6J mice for 20 generations (C57BL/6J-Tg(Thy1-APPK670N;M671L)23). All mice were kept under specific pathogen-free conditions. The experimental procedures were carried out in accordance with the veterinary office regulations of Baden-Württemberg (Germany) and with US federal guidelines, and all experiments were approved by the Institutional Animal Care and Use Committees.

Donor brain tissue

Human post-mortem brain samples (frontal cortex) were obtained at autopsy from histopathologically diagnosed AD patients (CERAD C/Braak stage VI; with extensive Aβ deposition in the frontal cortical tissue sample) and from a non-demented control patient (CERAD 0/Braak stage III-IV, with no Aβ deposition in the frontal cortical tissue sample) obtained from the brain bank affiliated with the University of Tübingen. Consent for autopsy was obtained from the legal representative in accordance with local institutional review boards. Prior to use, the human tissue was stored at 4°C or room temperature for 1-2 years (AD1: 2 years;

AD2 and Control: 1.5 years) in a PBS-neutral buffered 4.5% formaldehyde solution (Roti®-Histofix, Carl Roth, Karlsruhe, Germany). Prior to homogenization, residual formaldehyde was removed by rinsing the tissue under water for 2 h. Mouse brains were obtained from aged, Aβ-depositing APPPS1 tg mice (20-22 months old), APP23 tg mice (25-27 months old), and from age-matched, non-tg wildtype (WT) mice (Radde et al., 2006; Sturchler-Pierrat et al., 1997). After removal of the brain, the cerebellum and the lower brainstem were detached at the mesencephalic flexure and the forebrain was divided midsagittally into the two hemispheres. The left hemisphere was immediately fresh-frozen (unfixed) on dry ice and stored at −80°C until used. The right hemisphere was immersion-fixed for 48 h in PBS-buffered 4% formaldehyde solution (prepared by de-polymerization of 4% [w/v] paraformaldehyde in PBS) and then cryoprotected in 30% sucrose in PBS for an additional 48 h. The right hemibrains were then frozen on dry ice and stored at −80°C until use.

Extract preparation

Fixed and fresh-frozen tissues were homogenized at 10% [w/v] in sterile PBS at 4°C (4 x 10 seconds at 5500 rpm, each round separated by a 10 second pause) using the Precellys 24-Dual homogenizer (Bertin, Montigny-le-Bretonneux, France; 7 ml lysing tubes with 2.8 mm ceramic beads). Homogenates were centrifuged at 3000 x g for 5 min (4°C). The supernatants were aliquoted and immediately frozen. For all experiments, a 10% [w/v] extract was used unless otherwise stated.

Biochemical analyses

Fixed and fresh-frozen brain extracts were analyzed on NuPage Bis-Tris mini gels using NuPage LDS sample buffer and MES running buffer (Invitrogen). For Western blotting, samples were

semi-dry blotted onto a nitrocellulose membrane and then probed with antibody 6E10 to human-sequence Aβ (reactive to amino acid residues 1-16; Covance Research Products). Samples were visualized with chemiluminescence using SuperSignalWest Pico (Thermo Scientific). Synthetic Aβ from American Peptide was used for standardization.

Stereotactic injection of brain extracts

Male and female 3-4 month-old APP23 tg hosts were anesthetized using a mixture of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) in saline. 2.5 µl of brain extract was then infused bilaterally into the dorsal hippocampus (AP −2.5 mm, L ±2.0 mm, DV −1.8 mm) by stereotactic injection. Injection speed was 1.25 µl/min and the needle was slowly removed after being kept in place for an additional 2 min. The surgical area was cleaned with sterile saline and the incision was sutured. Mice were kept under infrared light for warmth and monitored until recovery from anesthesia.

Histology and immunohistochemistry

After an incubation period of 4 months, inoculated mice were perfused for 5 min with ice-cold PBS. Brains were removed and immersion-fixed in 4% formaldehyde in PBS for 48 h, and then placed in 30% sucrose in PBS for 2 d. Brains were frozen in 2-methylbutane cooled with dry ice and then serially cut into 25 µm-thick coronal sections using a freezing-sliding microtome. The sections were collected in cryoprotectant (35% ethylene glycol, 25% glycerol in PBS) and stored at −20°C until use. For immunostaining of Aβ, polyclonal antibody CN3 was used (Eisele et al., 2010). Sections were counterstained with Congo red according to standard protocols.

Stereological analysis

Total β-amyloid load was quantified on a CN3/Congo red-immunostained set of every 12th systematically sampled coronal section throughout the entire hippocampus. A microscope equipped with a motorized x-y-z stage coupled to a video-microscopy system and the Stereo Investigator software (MicroBrightField, Inc., Williston, VT) was used as previously described(Bondolfi et al., 2002). The investigators who performed the analysis were blind to the inoculation groups. The total β-amyloid load (percentage) was determined by calculating the areal fraction occupied by CN3- and Congo red-positive immunostaining in two-dimensional sectors (20x/0.45 objective).

Histological staining with pFTAA and spectral analysis

Coronal brain sections (25 μm) from inoculated mice were washed in PBS (3 x 10 min) and subsequently mounted on Superfrost slides. Sections were allowed to dry for 2 h at RT. Staining with pentamer formyl thiophene acetic acid (pFTAA; 1.5 mM in deionized water, diluted 1:1000 in PBS) was performed as previously described (Klingstedt et al., 2011). (Note that the trimeric polythiophene acetic acid (tPTAA) used in a previous study (Heilbronner et al., 2013) is no longer produced because of toxicity and stability issues, and has been replaced by pFTAA). Spectra were acquired on a Zeiss LSM 510 META (Axiovert 200M) confocal microscope (40x oil-immersion objective, 1.3 NA) equipped with an argon 458 nm laser for excitation and a spectral detector. Emission spectra were acquired from 470 nm to 695 nm. The spectra were collected from 3 matched regions of interest (ROIs) within 40 Aβ plaques per animal. The ratio of the intensity of emitted light at the green-shifted portion (492 nm) and red-shifted peak (599 nm) was used as a parameter for spectral distinction of different plaques. The peaks were

selected to maximize the spectral variations of pFTAA-stained APP23 and APPPS1 Aβ plaques obtained by this single wavelength excitation procedure in order to minimize exposure time.

In vitro aggregation assay

In brief, Thioflavin T was dissolved in water to generate a 200µM stock solution. Lyophilized recombinant Aβ1-40 peptide was dissolved to a stock concentration of 5mM in 100% DMSO. Prior to use, this stock was freshly diluted to 1mM in DMSO and sonicated for 15 min in a water bath, followed by 15 min centrifugation at 16,100 x g at room temperature. The supernatant was then further diluted to a concentration of 250 µM Aβ1-40 in a 50% DMSO stock solution. For kinetic measurement in the 96 well format, 1 µl of brain extract was incubated with 20µM Thioflavin T, 25µM Aβ1-40, 50mM phosphate and 150mM NaCl at 37°C. Using 96 well clear-bottom plates (Greiner, Bio-One), each brain homogenate was assayed in 8 sealed wells. Thioflavin T fluorescence at 480 nm was measured from the plate bottom every 30 min using a BMG Fluostar plate reader. Before each measurement, a double orbital shaking step at 500 rpm for 30 sec was performed. The increase in fluorescence over time was followed until the maximum signal was reached. Raw data were fitted and lag times determined with GraphPad Prism 5 as previously described (Nagarathinam et al., 2013).

2.4 Results and Discussion

Extract from formaldehyde-fixed AD brain tissue harbors β-amyloid inducing activity

Formaldehyde-fixed brain extract from two confirmed AD cases robustly induced Aβ deposition in young APP-tg mouse brains 4 months after intracerebral inoculation (Fig. 2.1). No such Aβ

deposits were observed following infusion of an extract from formaldehyde-fixed, non-demented human control brain. Fixed tissue extract from AD case 1 yielded more seeded Aβ deposition than did fixed extract from AD case 2 (Fig. 2.1C). Correspondingly, Aβ immunohistochemical staining indicated that AD case 1 had more Aβ-deposition (and in particular numerous dense-core plaques and significant Aβ-deposition in the vasculature) than did AD case 2. No Aβ-deposits was found in the control donor (Data not shown).

Extract from formaldehyde-fixed, β-amyloid-laden mouse brain tissue also induces β-amyloid deposition

To substantiate the amyloid-inducing activity of formaldehyde-fixed β-amyloid-containing human brain tissue, we repeated the inoculation experiments with brain extract from an aged (22 month-old) APPPS1 tg mouse. At this age, APPPS1 tg mice exhibit extensive cerebral β-amyloidosis (Radde et al., 2006).

For this experiment, the APPPS1 brain and a brain from an age-matched wildtype (WT) mouse were mid-sagittally divided into the two hemispheres. One hemisphere was immersion-fixed in formaldehyde while the other was fresh-frozen (unfixed) (Fig 2.2A). SDS-PAGE and subsequent Aβ-immunoblotting of the 3000 x g brain homogenate supernatants revealed ~20-fold less monomerized Aβ (and also total protein) in the extract from the formaldehyde-fixed tg hemisphere compared to the extract from the fresh-frozen tg hemisphere (Fig. 2.2B), presumably because less Aβ/protein was liberated from the tissue to enter the supernatant in the fixed samples (only a negligible fraction of the fixed tissue did not enter the gel). To adjust for the difference in the amount of Aβ in the extracts, the fresh-frozen brain material was diluted 1:20 to

approximate the Aβ concentration in the fixed brain extract prior to intrahippocampal injection of the extracts into APP23 tg mice. Aβ-immunostaining 4 months later revealed robust β-amyloid induction in the hippocampus of mice receiving the diluted (1:20) fresh-frozen tg brain extract (Fig. 2.2C). Extract from the formaldehyde-fixed tg hemisphere also induced Aβ deposition that was generally similar in pattern and morphology to that induced by the fresh-frozen extract (Fig. 2.2D). Stereological quantification of Aβ immunoreactivity revealed that the fixed tg mouse brain extract yielded less Aβ deposition than did the diluted fresh-frozen brain extract, although this difference was not statistically significant (Fig. 2.2G). No Aβ deposits were found after inoculation with extracts from fresh-frozen or fixed WT hemispheres (Fig. 2.2E and F).

The probable β-amyloid-inducing factor in fixed brain extracts is likely similar to that of fresh-frozen brain material, previously identified as Aβ species ranging in size from soluble oligomers to fibrillar Aβ (Langer et al., 2011; Meyer-Luehmann et al., 2006). However, because the denaturing conditions in SDS-PAGE tend to disrupt the native protein state and monomerize multimeric assemblies, the precise nature of the biologically active seeds in the fixed material (and whether they differ from seeds in the native state) remains to be determined.

Next, fixed and fresh-frozen extracts from three additional APPPS1 tg mice and WT mice (all 20-22 months old) were tested in an *in vitro* aggregation assay. The results confirmed both the *in vivo* seeding capability of formaldehyde-fixed brain homogenates as well as the reduced efficacy of the fixed material (Fig 2.3). Furthermore, as with APPPS1 tissue donors, extract from fixed brains of aged APP23 tg mice (25-27 months old) also displayed seeding activity *in vitro* (Fig. 2.3).

Formaldehyde fixation preserves the strain-like properties of seeded Aβ plaques

Aβ plaques in different APP-tg mouse lines vary in appearance and molecular architecture; whereas Aβ deposits in aged APP23 tg mice are fairly large with congophilic cores and diffuse penumbras, Aβ plaques in aged APPPS1 tg mice are small, compact, and highly congophilic (Heilbronner et al., 2013; Meyer-Luehmann et al., 2006). By cross-inoculation experiments, we previously showed that such Aβ plaque morphotypes can be maintained by seeded conversion, i.e., APPPS1 seeds injected into an APP23 host induce Aβ deposits reminiscent of endogenous Aβ plaques in aged APPPS1 mice, while APP23 seeds injected into an APP23 host induce Aβ deposits reminiscent of Aβ plaques in aged APP23 mice (Heilbronner et al., 2013).

To investigate whether this phenomenon of congruent templated conversion also applies to the fixed brain material, extracts from fixed and fresh-frozen APPPS1 and APP23 hemispheres were intracerebrally injected into young APP23 tg mice, respectively. Immunohistochemical analysis 4 months after inoculation revealed the expected Aβ morphotypes that result from injections of fresh-frozen brain extracts (i.e. Aβ plaques with diffuse penumbras for APP23 mice, and small, compact deposits for APPPS1 mice, Fig. 2.4A and B). However, fixed donor extracts from both APP23 and APPPS1 tg mice gave rise to Aβ plaques that were rather small and compact (Fig. 2.4C and D). Subsequently, luminescent conjugated oligothiophenes (LCOs) were used to further investigate whether the amyloid induced by extracts from fixed brains can be distinguished by their emission spectra, as previously shown for injections of fresh-frozen brain material (Heilbronner et al., 2013). Quantitative results revealed that pFTAA spectrally discriminates between Aβ deposits in mice that were injected either with fresh-frozen APP23 or fresh-frozen

APPPS1 brain extract (Fig. 2.4E), consistent with previous studies (Heilbronner et al., 2013). Spectral analysis of plaques induced by the injection of fixed APP23 or fixed APPPS1 brain extracts also showed a significant difference (Fig. 2.4E). These data indicate that formaldehyde fixation partially modifies the histological appearance of seeded Aβ plaque morphology, but at the same time maintains at least some of the basic conformational properties of the aggregated Aβ.

An important characteristic of prions is their resistance to inactivation by physical and chemical treatments that neutralize most microbes and viruses (Brown et al., 1990a; Pattison, 1965; Prusiner, 1998; Rutala and Weber, 2010). Although viruses vary in their sensitivity to formaldehyde treatment (Brown, 2001), the persistent infectivity of prions even after exposure to formaldehyde (Gordon, 1946) for months or years (Brown et al., 1986; Pattison, 1965; Pattison, 1972) was one of the earliest indications that the causative agent of scrapie was unlike conventional infectious agents (Pattison, 1965). Here we demonstrate that Aβ seeds, like prions, are resistant to inactivation by formaldehyde. Specifically, brain material containing aggregated Aβ from APP-tg mice (fixed for 48 h) or from human AD cases (fixed for up to 2 years) retained the capacity to seed Aβ deposition when infused into the brains of APP-tg host mice. Aβ seeds are not known to be infectious in the sense of facile transmissibility from one organism to another (Jucker and Walker, 2013). However, in light of previous studies showing that Aβ seeds remain functional following boiling (Meyer-Luehmann et al., 2006) or drying (Eisele et al., 2009), these findings further underscore their molecular commonalities with prions.

The seeding efficacy of fixed tissue was less than that of comparable unfixed tissue, possibly due to reduced liberation of Aβ from fixed samples during homogenization, as shown by immunoblot analysis of the extracts (Fig. 2.2B). It is also possible that fixation additionally diminishes the

inductive efficacy of Aβ seeds by corrupting their molecular structure, although the LCOs indicate that formaldehyde fixation largely preserves the strain-like properties of the induced amyloid. The loss of diffuse Aβ immunostaining in the penumbras of plaques that were induced with extract from the fixed compared to fresh-frozen APP23 brains may indicate that various Aβ species are differentially sensitive to formaldehyde fixation.

Aβ in fixed tissue is partially resistant to digestion by proteinase K (unpublished data), suggesting that fixation may act to immobilize and shield Aβ seeds from proteolytic degradation. Interestingly, tissue fixation by formaldehyde can protect prions against inactivation by autoclaving (Brown et al., 1990a; Taylor and McConnell, 1988), though denaturation with formic acid effectively neutralizes prions in fixed tissue (Brown et al., 1990b; Taylor et al., 1997). Formic acid also negates the amyloidogenic capability of Aβ seeds in unfixed tissue (Meyer-Luehmann et al., 2006). Given the effectiveness of formic acid against prion infectivity, it seems likely that formic acid also neutralizes Aβ seeds in fixed tissue, but this possibility remains to be tested. Importantly, the resistance of prions to standard methods of inactivation has prompted improvements in the sterilization of medical instruments and general handling practices for tissue suspected of harboring prions (Rutala and Weber, 2010). As a result, iatrogenic transmission of prion disease to humans has essentially ceased (Brown et al., 2012).

Conclusions

Our experiments show that Aβ seeds in brain tissue extracts retain the ability to induce β-amyloidosis even after prolonged (2 years) exposure to formaldehyde. Furthermore, the formaldehyde-fixed seeds largely retained their conformational templating capacity, as the nascent amyloid deposits replicated the spectral properties of the parental seeds, at least at the binding sites of conformation-sensitive oligothiophene ligands. The discovery that both the self-

propagating activity and strain-like features of aggregated Aβ are maintained in fixed tissue further supports the incorporation of Aβ seeds into the broad conceptual framework of prions. In addition, the stability of the seeds in fixed material can now be exploited to establish the relationship between the molecular architecture of Aβ and the variable clinical features of AD, even in archived, formalin-fixed brain samples. Although Aβ seeds can persist in fixed tissue, the implications for standard laboratory practice are uncertain. Currently the weight of evidence – albeit mostly circumstantial – argues against the exogenous infectivity of Aβ seeds in AD (Irwin et al., 2013; Jucker and Walker, 2013). Rather, AD appears to arise spontaneously due to the endogenous emergence and autopropagation of pathogenic protein seeds. The present findings highlight the extraordinary robustness of Aβ seeds; once they gain a foothold in the brain, their resistance to destruction facilitates the formation and endogenous spread of new seeds, thereby sustaining the disease process.

Figures

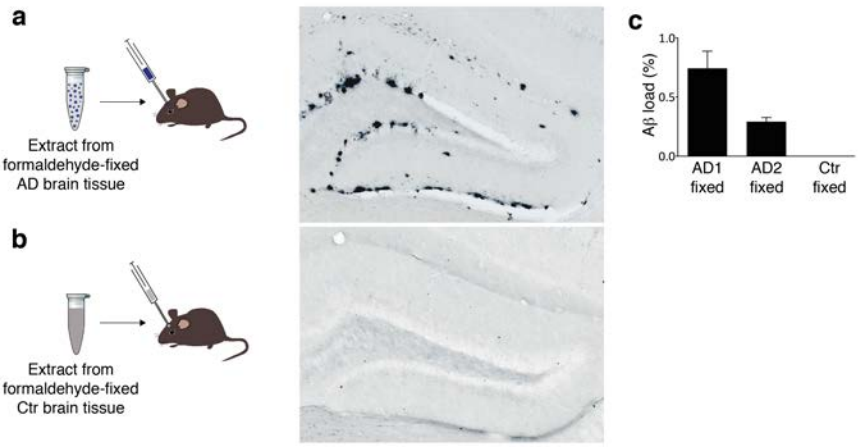

Figure 2.1. *Formaldehyde-fixed brain tissue from AD patients induces cerebral β-amyloidosis in APP23 transgenic mouse hosts.* (**a, b**) Young 3-4 month-old APP23 transgenic (tg) mice were intracerebrally inoculated with extracts of formaldehyde-fixed tissue from two AD cases (AD1 and AD2) or from a non-demented human control (Ctr) case. Four months after intrahippocampal extract injection (2.5 µl), Aβ-deposition was induced in the hippocampus of APP23 tg mice receiving AD extract **(a)**. No Aβ-deposits were induced by the control brain extract **(b)**. **(c)** Stereological quantification of the percent area of the hippocampus occupied by immunoreactive Aβ (Aβ load) induced by extract from the two AD cases and the control case (n = 3−6 mice/group, mean ± SEM, scale bar: 200 µm).

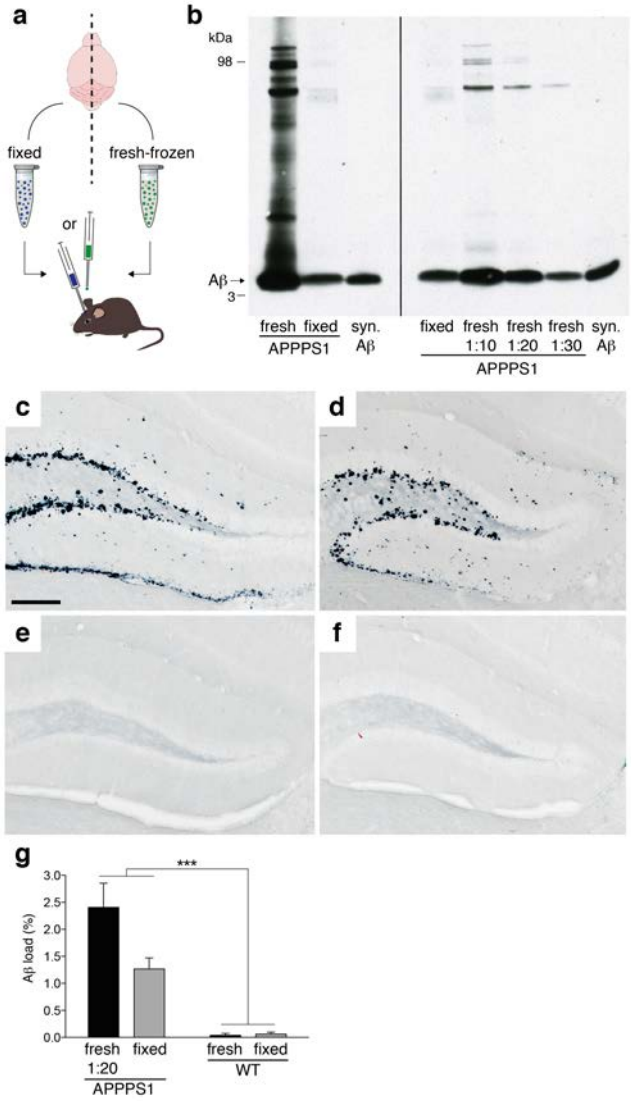

Figure 2.2. *Formaldehyde-fixed brain tissue from an aged APPPS1 mouse donor induces cerebral β-amyloidosis in APP-tg mouse hosts.* (**a**) Schematic diagram of the tissue preparation protocol in which hemibrains from aged APPPS1 tg mice and non-tg wildtype mice (WT) underwent formaldehyde fixation ("fixed") or were fresh-frozen ("fresh"). (**b**) Immunoblot analysis with an antibody specific to human Aβ (6E10) reveals approximately 20-fold less recoverable (monomerized) Aβ in the brain extract from the fixed tg hemisphere compared to the fresh-frozen tg hemisphere. (**c-f**) *In vivo* seeding capacity of fresh-frozen or fixed brain extracts

following intrahippocampal injection into pre-depositing 3-4 month-old APP23 tg mice. Brains were immunohistologically analyzed 4 months later. Robust Aβ deposition was found in the hippocampal formation after inoculation with extract from the fresh-frozen (1:20 diluted) tg brain (**c**). Aβ deposition also was induced by brain extract from the formaldehyde-fixed tg mouse brain (**d**). Extracts from both fresh-frozen (**e**) and fixed (**f**) WT brains did not induce Aβ deposits. **(g)** Stereological quantification of the percent area of the hippocampus occupied by immunoreactive Aβ (Aβ load) revealed that the deposition induced by extract from the fixed tg hemibrain was about half of that achieved with the 1:20-diluted tg extract from the fresh-frozen hemibrain. ANOVA (genotype of donor material x tissue preparation) revealed a significant effect of the donor genotype ($F(1, 16) = 33.64$; $p<0.001$) but no significant effect of the tissue preparation ($F(1, 16) = 3.282$; $p = 0.089$) or interaction ($F(1, 16) = 3.582$; $p = 0.077$) ($n = 4−6$ mice/group; mean ± SEM, scale bar: 200 μm).

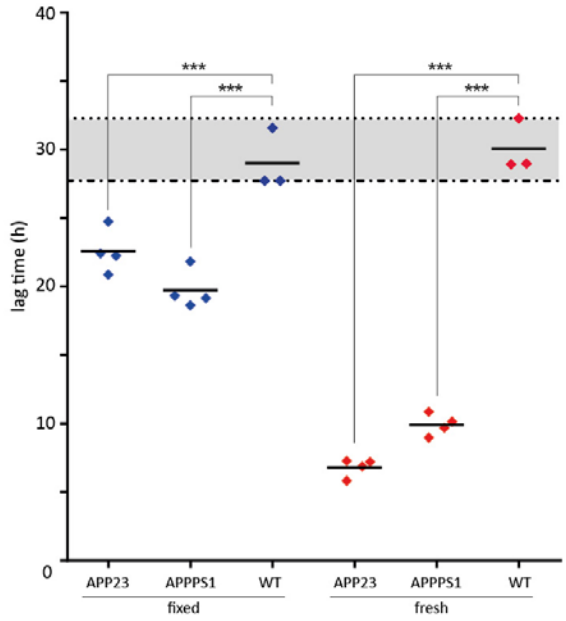

Figure 2.3. *Formaldehyde-fixed brain material from APP-transgenic mice harbors in vitro seeding capacity.* Fibrillization kinetics of recombinant Aβ1–40 were monitored by incorporation of Thioflavin T (ThT). Lag times were determined as measures of the seeding capacity of the extracts. Both APP23 brain tissue and APPPS1 brain tissue show reduced *in vitro* seeding activity in response to formaldehyde fixation (n = 4/group). Note that the fresh-frozen extracts were not diluted in this experiment (compare also to Fig. 2). However, extracts of both fixed APP23 and fixed APPPS1 mouse brains still show strong seeding activity compared to fixed (or fresh-frozen) wildtype (WT) brain extracts (n = 3/group). Each data point represents the mean lag time of an individual brain extract determined by measurement of eight technical replicates, with seeding by all of the extracts measured twice, with the exception of extracts from three donor mice that were measured only once. The mean of each group is indicated by a black line. ANOVA (genotype of donor material x tissue preparation with matched fixed vs. fresh values) revealed a significant effect of the donor genotype ($F(2, 8) = 124.8$; $p<0.001$), tissue preparation ($F(1, 8) = 759.9$; $p<0.001$) and interaction ($F(2, 8) = 256.1$; $p<0.001$). Subsequent

Tukey´s multiple comparison tests revealed that lag times associated with both fixed APP23 and fixed APPPS1 mouse brain extracts were shorter compared to lag times in the presence of fixed WT brain extracts (all *p*s<0.001). Similarly, the lag times yielded by fresh-frozen APP23 and APPPS1 mouse brain extracts were significantly shorter compared to those associated with fresh-frozen WT brain extracts (all *p*s<0.001).

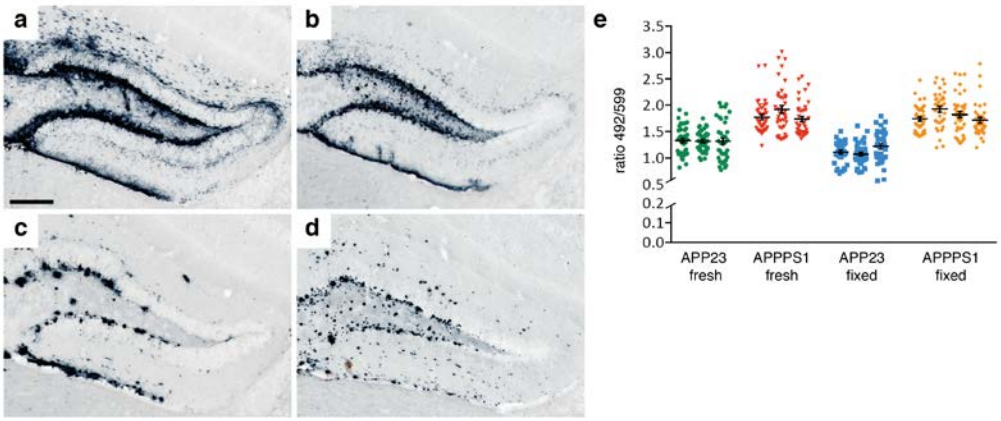

Figure 2.4. *Formaldehyde fixation preserves strain-like properties of seeded Aβ plaques.* Brain extracts from aged APPPS1 or APP23 tg donor mice (the extracts were from two donor mice each) were intracerebrally injected into pre-depositing, 3-4 month-old APP23 tg host mice. Brains were analyzed 4 months after inoculation using Aβ immunostaining. (**a-d**) The extract of the fresh-frozen APP23 brain tissue induced a more diffuse pattern of Aβ deposition (**a**), whereas the extract of the fresh-frozen APPPS1 brain tissue induced a more punctate pattern of Aβ deposition (**b**). Injection of extracts from formaldehyde-fixed brain tissue induced a diffuse pattern for APP23 (**c**) and a punctate pattern for APPPS1 (**d**) donor mice (scale bar: 200 µm). (**e**) Spectral properties of the induced Aβ deposits using luminescent conjugated oligothiophenes (pFTAA). For quantitative analysis, the ratio of the intensity of the emitted light at 492 nm and 599 nm was calculated (see Methods). Each dot represents one Aβ plaque. The mean and SEM are indicated for each animal (n = 3−4/group). ANOVA (genotype of donor material x tissue preparation) revealed a significant effect of the donor genotype ($F(1, 9) = 165.6$; $p<0.001$) but no significant effect of the tissue preparation ($F(1, 9) = 4.856$; $p = 0.055$) or interaction ($F(1, 9) = 3.947$; $p = 0.078$).

Chapter 3

Transport of Aggregated Aβ and Other Cargo from Periphery to Brain by Circulating Monocytes[2]

[2] This chapter is reproduced with minor changes from the original publication: Cintron A, et al., Brain Research 2015 *In Press*.

3.1 Abstract

The misfolding and aggregation of the Aβ peptide – a fundamental event in the pathogenesis of Alzheimer's disease – can be instigated in the brains of experimental animals by the intracranial infusion of brain extracts that are rich in aggregated Aβ. Recent experiments have found that the peripheral (intraperitoneal) injection of Aβ seeds induces Aβ deposition in the brains of APP-transgenic mice, largely in the form of cerebral amyloid angiopathy. Macrophage-type cells normally are involved in pathogen neutralization and antigen presentation, but under some circumstances, circulating monocytes have been found to act as vectors for the transport of pathogenic agents such as viruses and prions. The present study assessed the ability of peripheral monocytes to transport Aβ aggregates from the peritoneal cavity to the brain. Our initial experiments showed that intravenously delivered macrophages that had previously ingested fluorescent nanobeads as tracers migrate primarily to peripheral organs such as spleen and liver, but that a small number also reach the brain parenchyma. We next injected CD45.1-expressing macrophages from donor mice intravenously into CD45.2-expressing host mice; after 24 hours, analysis by fluorescence-activated cell sorting (FACS) and histology confirmed that some CD45.1 monocytes enter the brain, particularly in the superficial cortex and around blood vessels. When the donor macrophages are first exposed to Aβ-rich brain extracts from human AD cases, a subset of intravenously delivered Aβ-containing macrophages migrate to the brain. These experiments indicate that, in mouse models, circulating macrophages are potential vectors by which exogenously delivered, aggregated Aβ travels from periphery to brain, and more generally support the hypothesis that macrophage-type cells can participate in the dissemination of proteopathic seeds.

3.2. Introduction

The aggregation of the beta-amyloid peptide (Aβ) in the brain is an early and integral event in the pathogenesis of Alzheimer's disease (Hardy and Selkoe, 2002; Holtzman et al., 2011). Aβ normally exists in a structurally unfolded (intrinsically disordered) state, but in its pathogenic form, Aβ becomes rich in β-sheet structure and induces the misfolding and subsequent self-assembly of other Aβ molecules. These durable, prion-like multimeric seeds instigate the formation of senile plaques and cerebral β-amyloid angiopathy as monomeric Aβ is recruited into the β-sheet-rich deposits (Jucker and Walker, 2013; Walker et al., 2006; Walker and LeVine, 2012). We and others have found that intracranial injections of Aβ multimers seed Alzheimer-like pathology in Aβ-precursor protein (APP) transgenic rodent models (Duran-Aniotz et al., 2014; Eisele et al., 2009; Fritschi et al., 2014; Hamaguchi et al., 2012; Kane et al., 2000; Langer et al., 2011; Meyer-Luehmann et al., 2006; Morales et al., 2012a; Morales et al., 2012b; Rosen et al., 2012; Stohr et al., 2012; Stohr et al., 2014; Watts et al., 2011; Watts et al., 2014).

Within the brain, the proteinaceous lesions that characterize Alzheimer's disease and other protein misfolding disorders appear to propagate among interconnected brain areas, suggestive of the dissemination of seeds by means of axonal transport (Boluda et al., 2015; Clavaguera et al., 2009; Clavaguera et al., 2014; Guo and Lee, 2011; Guo and Lee, 2014; Hyman, 2014; Liu et al., 2012; Walker and LeVine, 2012; Walker et al., 2013; Ye et al., 2015). Other studies have shown that the injection of Aβ multimers into the peritoneal cavity of APP-transgenic mice can seed Aβ deposition in the brain, particularly in the form of cerebral β-amyloid angiopathy (Eisele et al., 2010; Eisele et al., 2014). While the evidence currently favors axonal transport as a key mode of lesion propagation within the nervous system, the mechanism by which Aβ seeds are transported

from periphery to brain remains uncertain. The preponderance of amyloid angiopathy in the forebrain of intraperitoneally seeded APP23 transgenic mice (Eisele et al., 2010) suggests the possibility that the seeds reach the brain via the vasculature (Eisele et al., 2014). Furthermore, the presence of Aβ within circulating macrophages of these mice implicates these cells as possible vectors for the transport of seeds from periphery to brain (Eisele et al., 2014). The poor ability of microglia to degrade amyloid fibrils further supports the idea that aggregated Aβ may remain intact in macrophages for a long period of time (Frackowiak et al., 1992), but the evidence for the entry of Aβ-laden macrophages from the circulation into the brain remains indirect.

As a component of the innate immune system, macrophages normally serve to phagocytose and degrade exogenous pathogens such as microbes, and they also present antigen to cells of the adaptive immune system (Alberts, 2002). However, in some instances macrophages have been found to ingest and disseminate pathogens intact (Ferreira et al., 2010; Johnson et al., 2010; Kirby et al., 2009; Tanaka et al., 2012), thereby contributing to the disease process. In the brain, microglia are resident macrophages that originate from the yolk sac early in embryogenesis and replenish themselves by self-replication (Prinz et al., 2011). Particularly in disease states, circulating (hematogenous) monocytes can differentiate into macrophages in the brain, where they become part of local cellular networks (Mildner et al., 2007; Priller et al., 2001). Studies in which the blood-brain barrier is disrupted by irradiation demonstrate an increased infiltration of circulating monocytes into the brain (Mildner et al., 2007). Furthermore, disease models of multiple sclerosis show that hematogenous monocytes enter the brain and are responsible for stripping myelin from axons (Lampert, 1978). Activation of circulating monocytes in an

Alzheimer's disease model resulted in the increased phagocytosis of β-amyloid, thereby reducing the number of senile plaques (Shaftel et al., 2007; Town et al., 2008). Thus, in disease states it is clear that circulating monocytes are able to enter the brain, but it is thought that few, if any, circulating monocytes cross the intact blood-brain barrier to enter the healthy brain (Kroll and Neuwelt, 1998; Prinz et al., 2011; Zhang and Pardridge, 2001). In the present study, we tested the hypothesis that monocytes are able to phagocytose and convey cargo from the peritoneal cavity to the brain in healthy mice. We found that limited numbers of these cells can enter the brain parenchyma, and thus could act as vectors for the transport of proteopathic seeds.

3.3. Methods

3.3.1 Subjects. Non-transgenic C57BL/6 mice served as subjects. For the FACS/histology experiments, wild-type, inbred C57BL/6 mice expressing the hematopoietic cell marker CD45.2 (B6[CD45.2]) mice served as hosts, and congenic B6[CD45.1] mice (B6.SJL-Ptprca Pepcb/BoyJ; The Jackson Laboratory) served as donors. CD45 is a pan-leukocytic antigen that has two differentiable allelic forms in the two murine lines; as such, CD45.1-expressing cells can be readily distinguished from CD45.2-expressing cells. Mice were housed in small groups under standard conditions at a temperature of 22°C and a 12 h light/dark cycle with ad libitum access to food and water. All experimental procedures were carried out in accordance with US federal guidelines, and were approved by the Emory Institutional Animal Care and Use Committee (IACUC).

3.3.2. Characterization of lavaged leukocytes by fluorescence-activated cell sorting (FACS)

To characterize the cell population that was induced in the mice, intraperitoneal cells were collected by lavage from C57/BL6 mice (Ray and Dittel, 2010) and gently centrifuged at 1000 x g for 5 min. The supernatant was removed, the red blood cells lysed in diH2O, and the leukocytic fraction was re-sedimented at 1000 x g for 5 min and re-suspended in buffered physiological saline. This fraction was incubated with antibodies to CD11b and F4/80 (macrophage-specific antigens), Ly6G and Ly6C (transiently expressed on monocytes in the bone marrow), B220 (a pan B-cell marker) and CD3 (primarily expressed by T-cells) for 25 minutes on ice in CD16/CD32 mouse BN Fc Block (BD Pharmingen) (chromogens FITC and phycoerythrin [PE]). After washing, fixing buffer was added and the pellet was washed again. The pellet was resuspended in FACS buffer (PBS+ 2% FBS) and analyzed by FACS for expression of CD11b and F4/80 on double-positive cells. The cells were gated by size (forward scatter) and density (side scatter), and then plotted according to CD11b and F4/80 expression. The resulting population was further gated by level of expression. CD11b-intermediate and F4/80-negative expressing cells were gated and plotted according to Ly6G and Ly6C expression. CD11b-negative and F4/80-negative expressing cells were gated and plotted according to B220 and CD3 expression. In this way, the composition of cells in the lavage fraction can be quantified (see Results).

3.3.3 Trafficking of macrophages bearing fluorescent nanobeads Our first objective was to assess the systemic distribution of macrophages that had ingested fluorescent nanobeads as tracer cargo. Five wild-type C57/BL6 mice received intraperitoneal (i.p.) injections of red fluorescent nanobeads (SPHERO™ Fluorescent Nile Red Particles; 2 µl of 1% nanobeads [w/v], 0.53µm diameter, Spherotech, Lake Forest, IL, USA) along with 100µl of thioglycolate to elicit

macrophages on day 0. On day 3, mice were given a second injection of nanobeads, and animals were then sacrificed on day 4. In one group of mice (n=5), the nanobead-containing macrophages were analyzed in the mice that received the nanobead injections, i.e., the tracked macrophages originated endogenously within the same mouse. In a separate group of host mice (n=10), bead-laden macrophages were prepared in donor mice as described above, and then harvested and infused intravenously (i.v.) into host mice (exogenous macrophages).

Leukocytes were collected by lavage (Ray and Dittel, 2010) and gently centrifuged at 1000 x g for 5 min. The supernatant was removed, the red blood cells lysed in diH2O, and the leukocytic fraction re-sedimented at 1000 x g for 5 min, re-suspended in buffered physiological saline, and counted using a hemocytometer. The cells were assessed for viability in a test sample by trypan blue exclusion. In all cases, a minimum of 75% of the cells for infusion were viable. In each host mouse, 5x106 viable cells in 250µl physiological saline were slowly infused intravenously via the tail vein. Following an incubation period of either 4 days or 4 weeks, the host mice were perfused with 200mL of cool (4-8oC) physiological saline and the following tissues collected: Brain, spleen, liver, lung, heart, kidney, pancreas, and blood.

3.3.4 FACS analysis of CD45.1-expressing exogenous macrophages transferred to B6[CD45.2] host mice Our second objective was to determine the proportion of exogenous macrophages that enter the brain from the bloodstream following infusion of the cells into host mice. A total of 15 mice served as hosts in two separate experimental runs. Macrophages were elicited in the peritoneal cavity of B6[CD45.1] donor mice by i.p. injection of thioglycolate on Day 1 and Day 3, and the cells were collected by lavage on day 4 (Ray and Dittel, 2010), as described above.

The CD45.1-bearing cells (5x106 cells/250µl) were then slowly infused i.v. into CD45.2-expressing host mice. 24 hours later, the host mice were transcardially perfused with 200mL of cool (4-8oC) physiological saline under deep Nembutal anesthesia.

For FACS analysis the whole brain and spleen were separately processed and the blood vessels depleted from the homogenates (D'Alessandro et al., 2013; Pertoft, 2000) as follows: The organs were homogenized over a 100µm mesh nylon sieve, lysed in collagenase IV (Worthington), and mononuclear cells (along with a small population (~10%) of other leukocytes; see Figure 3.1) were obtained by density-gradient centrifugation using Percoll (Amersham) solutions (40% and 70%). Cells were incubated with antibodies to CD45 (a pan-hematopoietic cell antigen) and CD45.1 for 25 minutes on ice in CD16/CD32 mouse BN Fc Block (BD Pharmingen) (fluorochromes FITC and phycoerythrin [PE] respectively). After washing, fixing buffer was added and the pellet was washed again. The pellet was resuspended in FACS buffer (PBS + 2% FBS) and analyzed by FACS for expression of CD45.1 on CD11b/CD45 double-positive cells. The cells were gated by size (forward scatter) and density (side scatter), and then plotted according to CD45 and CD45.1 expression. In this way, the entry of exogenous macrophages from the donor mouse can be assessed quantitatively in the brain and spleen of the host mouse.
To further characterize the cells and tissues, induced intraperitoneal cells were harvested from donor mice on day 4 and plated in EMEM (Eagle's Minimum Essential Medium). To promote growth we used 10ng/ml of macrophage colony-stimulating factor (M-CSF). Murine macrophages were isolated according to the protocol of Fortier and Falk (Fortier and Falk, 2001). The cells were maintained for 3 days before they were dried on slides and processed for immunocytochemistry.

For histology, the brains of B6[CD45.2] host mice were sectioned at 40μm thickness (as described below) and stained with antibodies 6E10 to human Aβ, laminin (blood vessels), and CD45.1 (transferred hematopoietic cells (see section 4.6 below for antibody details). Tissues were counterstained with a fluorescent nuclear marker (4',6-diamidino-2-phenylindole [DAPI]).

3.3.5 Trafficking of macrophages bearing Aβ-rich brain extract For our third objective, we sought to assess the fate of macrophages that had specifically ingested Aβ-rich brain extract to determine whether these cells can subsequently enter the host brain. The extract was prepared as described previously (Kane et al., 2000; Meyer-Luehmann et al., 2006). In brief, neocortical tissue from a histopathologically verified AD case was homogenized at 10% (w/v) in PBS at 4°C, followed by brief sonication, also at 4°C. The homogenate was centrifuged at 3,000×g for 5 minutes at 4°C, and the supernatant was aliquoted and immediately frozen at -80oC until use. Donor B6[CD45.1] mice received i.p. injections of either thioglycolate alone (resulting cells infused into 6 host mice) or thioglycolate and 250μl of 10% AD brain extract (resulting cells infused into 9 host mice) on day 0 and day 3, and were sacrificed on day 4. Macrophages were collected by lavage, processed, counted, and slowly infused into B6[CD45.2] host mice as described above. After 24 hours, the host mice were perfused with cool (4-8^{o}C) physiological saline and the brain and systemic organs collected for IHC and FACS analysis.

3.3.6 Immunohistochemistry All tissues were immersion-fixed in buffered, 4% de-polymerized paraformaldehyde for 24 hours followed by cryoprotection in buffered 30% sucrose. The tissues were frozen, sectioned at 40μm thickness on a Leica CM3050 S cryostat, and mounted onto

slides. Lavage samples of cells were dried onto slides and fixed with methanol. Selected specimens were immunostained with the following antibodies: 6E10 (1:5,000) (Covance), a mouse monoclonal antibody to human-sequence Aβ with an epitope at residues 3-8; rabbit polyclonal antibodies R361 and R398 (both at 1:15,000) (courtesy of Dr. Pankaj Mehta of the Institute for Basic Research on Developmental Disabilities, Staten Island, NY) to Aβ32-40 and Aβ33-42, respectively; and antibodies to CD45 (1:5000), CD45.1(1:1000)(BD Pharmingen), and laminin (1:1000)(Abcam ab11575). Specimens were washed in PBS, permeabilized using PBST (PBS and 0.2% Tween), and blocked for 1 h in 2% serum in PBST. Primary antibodies were added to PBST containing 2% serum and incubated with the cells or tissues overnight at 4°C while gently rocking. Secondary antibodies were added to PBST containing 2% serum and incubated with the specimens for 1.5 h at room temperature. In addition, some samples were counterstained with DAPI or hematoxylin. Cells and tissues were examined with a Leica DMLB microscope or an Olympus FV1000 / TIRF inverted confocal microscope.

3.4. Results

3.4.1. Characterization of lavage cell-types by FACS

To characterize the cells collected by lavage, FACS analysis was performed using antibodies specific for macrophages and lymphocytes (Figure 3.1). The analysis showed 3 distinct populations based on the expression of specific markers: CDllb-high/F4/80-high; CDllb-intermediate/F4/80-negative; and CDllb-negative/F4/80-negative. Roughly 30 percent of the lavage consisted of large peritoneal macrophages (LPM), which are characterized by high expression of CDllb and F4/80 (Ghosn et al., 2010). The cell population characterized by

intermediate CDllb and negative F4/80 expression was gated and investigated for Ly6G and Ly6C expression. The majority (94.3 percent) of the cells were Ly6G-negative and Ly6C-negative, indicating that these cells are small peritoneal macrophages (Ghosn et al., 2010; Gordon and Taylor, 2005; Rose et al., 2012). The CDllb-negative/F4/80negative cells were gated and investigated for expression of B220 (B-cells) and CD3 (T-cells) (Rodig et al., 2005). Of this subpopulation, 80.5 percent of the cells were B-cells and 15.7 percent were T-cells. Overall, this analysis indicates that the lavage cells consist of ~60% macrophages (LPM and SPM) and ~30% lymphocytes (most of which are B-cells).

3.4.2. Systemic distribution of labeled macrophages

To determine the general distribution of macrophages that had previously ingested fluorescent nanobeads, we assessed nanobead-labeled cells histologically in the brain and systemic organs. Host mice received either i.p. injections of nanobeads (ingested by endogenous macrophages), or i.v. injections of exogenous, nanobead-laden macrophages harvested from the peritoneal cavity of donor mice. In both groups, the systemic distribution of macrophages was similar, i.e., both endogenous and exogenous macrophages had comparable patterns of distribution in the body. Our analysis then focused on the mice receiving exogenous macrophages. At both four days and four weeks post-infusion of cells, bead-laden phagocytes were primarily found in the spleen (Figure 3.2), liver, and kidney of the host mice, and (in much smaller quantities) in lung and blood (not shown). The spleen in particular contained more nanobeads than any other structure, consistent with its role in the storage and deployment of monocytes (Swirski et al., 2009). In addition, a small number of bead-containing macrophages (<50 per 40μm section at 4 days and <10 per 40μm section at 4 weeks) were detected in grey matter structures and superficial cortex of the brain (not pictured).

3.4.3. A limited number of exogenous macrophages enter the intact brain

To quantify more precisely the exogenous macrophages that enter the intact brain in our paradigm, macrophages were elicited in the peritoneal cavity of CD45.1-expressing donor mice and then infused i.v. into CD45.2-expressing host mice. Twenty-four hours later, the host brains were gently homogenized and the blood vessels depleted from the homogenates to eliminate cells that might have remained within the vascular lumen following perfusion. For comparison, we also assessed the population of exogenous macrophages that had migrated to the spleen at the same time point. A small number of donor macrophages were detectable in the brain parenchyma 24 hours after i.v. infusion, and, as expected, many more donor cells had migrated to the spleen (Figure 3.3).

3.4.4. Exogenous macrophages containing Aβ seeds enter the intact brain

Having established that macrophages can enter the brain from the bloodstream, even after ingestion of nanobeads, we next asked whether exogenous macrophages that had ingested Aβ-rich brain extract also can enter the brain. First, to confirm the ingestion of human Aβ by peritoneally elicited macrophages, wild-type mice received i.p. injections of AD brain extract containing ample aggregated Aβ, and after 4 days the cells were plated on coverslips and immunostained with antibody 6E10 to human-sequence Aβ, which does not recognize murine Aβ (Kim et al., 1988; Otvos et al., 1993). Confocal microscopy verified that macrophages ingest detectable quantities of the Aβ-laden brain extract (Figure 3.4). CD45.1 macrophages containing human Aβ were then infused i.v. into B6[CD45.2] host mice; after a 24 hour incubation period, immunohistochemistry revealed Aβ-containing cells in the superficial neocortex and subarachnoid space (Figure 3.5). Additionally, some Aβ-immunoreactive cells were localized

around blood vessels (Figure 3.6), but we did not detect CD45.1 in these perivascular cells. The reason for the absence of the donor marker in perivascular cells is not clear, but suggests that the Aβ may have been transferred from exogenous donor cells to host macrophages.

3.5 Discussion

Both circulating monocytes and microglial cells are, under the appropriate circumstances, involved in the immune response in the brain. These two types of innate immune cells are among the first to respond to pathological insults; they help to repair physical trauma, defend against pathogens, and remove debris such as the remnants of dead cells (Flannagan et al., 2009; Gregory and Devitt, 2004). While they share some common functions, monocytes and microglia have distinct origins (Epelman et al., 2014; Gautier et al., 2012; Hettinger et al., 2013; Murray et al., 2014) and they monitor different tissue domains within the brain. When alerted to a pathological event in the CNS, the resident microglia are responsible for initiating the immune response, which can include the recruitment of circulating monocytes (Gate et al., 2010). The role of peripheral monocytes that enter the brain remains somewhat ambiguous, in part because they can be difficult to distinguish from resident microglia (Prinz et al., 2011).

Circulating monocytes enter the brain under various pathological conditions, including immunosuppression (Bauer et al., 1995) and irradiation injury (Mildner et al., 2007). In addition, monocytes enter the brain when the blood-brain barrier is physically compromised, for example in cases of stroke (Schilling et al., 2003; Tanaka et al., 2003) or head injury (Szmydynger-Chodobska et al., 2012). Our study indicates that a small but consistent subset of exogenous

circulating macrophages enter the intact, healthy brain, and this was so whether they contained nanobeads, Aβ-rich brain extract, or no specific cargo.

Histological evidence that hematogenous monocytes can enter the brains of healthy mice was confirmed by FACS analysis. The brain samples for FACS underwent perfusion and vascular depletion to minimize the possibility that the exogenous cells detected by the cell sorter were within the blood vessels of the brain, rather than on the parenchymal (abluminal) side of the blood-brain barrier (Triguero et al., 1990). However, we cannot rule out the possibility that some of these cells were within the vasculature, for example as dislodged marginating cells (van Furth and Sluiter, 1986), or in areas lacking capillary endothelial tight junctions such as the circumventricular organs or choroid plexus. However, in our histological samples we did not detect exogenous cells in these regions, whereas they were evident in the brain parenchyma, indicating that some circulating cells do cross from blood to brain.

FACS analysis of cell types within the lavage sample demonstrated the presence of both macrophages (60%) and lymphocytes (30%). We believe the macrophages are most likely to be responsible for the transport and dissemination of Aβ seeds because they are the principal cell type that is specialized for the removal of dying/dead cells and cellular debris (Perry et al., 2010). Some evidence suggests that B-cells can be phagocytic in various disease states (Borrello and Phipps, 1996), suggesting that they also could contribute to the transport of Aβ seeds. In contrast to seed dissemination, the presence of lymphocytes (which were primarily B-cells in the lavage sample) could result in the presentation of antigens to Aβ. Macrophages also present antigen (Unanue, 1984), so it is important to consider the possibility that engagement of the

adaptive immune response could result in antibody production and a decrease in cerebral Aβ deposition. Long-term studies of the impact of exogenous, Aβ-containing immune cells will be needed to address this question.

Taken together, our studies indicate that macrophages are able to take up and transport material from the periphery to the brain through the vasculature. In this light, the findings support the hypothesis that peripheral macrophages are a source of amyloidogenic Aβ seeds that traffic to the brain from the peritoneal cavity in an experimental seeding model (Eisele et al., 2010; Eisele et al., 2014). The localization of cells adjacent to cerebral blood vessels further supports this conclusion, in that the Aβ that deposits in the brains of peripherally seeded APP23 mice is mainly in the form of amyloid angiopathy (Eisele et al., 2009; Eisele et al., 2010).

In humans, there is currently no evidence that Alzheimer's disease can be induced by peripheral seeds (Irwin et al., 2013; Jucker and Walker, 2013). However, the fundamental molecular mechanisms by which proteopathic seeds arise and spread are unlikely to differ among species. At the cellular level, the role of macrophages – both central and peripheral – in the deposition of Aβ remains uncertain (Gate et al., 2010). Some studies have indicated that these cells can reduce the amount of Aβ in the brain (Simard et al., 2006), whereas others indicate that they promote deposition (Akiyama et al., 2000). It is possible that both processes are at play, depending on the circumstances. For example, the removal of senile plaques in Alzheimer patients who had been immunized against Aβ was associated with an increase in the amount of cerebral Aβ angiopathy (Boche et al., 2008; Ferrer et al., 2004; Masliah et al., 2005; Nicoll et al., 2003), suggesting that Aβ seeds were taken up and transported from the plaques to the vascular wall by macrophages

(Boche et al., 2008; Masliah et al., 2005). Our model supports a general role of macrophages in the uptake and dissemination of Aβ seeds; how the seeds exit the cells and stimulate Aβ deposition in the vascular wall remains to be determined.

Another potential mechanism by which proteopathic seeds might reach the brain is axonal transport. However, in a preliminary experiment, we found that the intraperitoneal injection of the tracer fluorogold led to retrogradely labeled cells only in the dorsal motor nucleus of the vagus nerve (data not shown), an area that does not exhibit aggregated Aβ in peripherally seeded models. Even so, we cannot yet rule out a role of neuronal transport in the translocation of Aβ seeds from periphery to brain. Prions, for example, can reach the CNS from the periphery by neuronal transport (Bartz et al., 2005; McBride et al., 2001; Sigurdson et al., 2001; van Keulen et al., 1999). Furthermore, within the CNS, cellular uptake and axonal transport mechanisms are implicated in the systematic spread of a number of pathogenic protein aggregates, including prions (Aguzzi, 2003; Borchelt et al., 1994; Buyukmihci et al., 1983; Liberski et al., 1990; Liberski et al., 2012; Scott et al., 1992), Aβ (Hallbeck et al., 2013; Hamaguchi et al., 2012; Lee et al., 2010; Saper et al., 1987; Ye et al., 2015), tau (Braak and Del Tredici, 2011; Clavaguera et al., 2009; Holmes et al., 2014) and α-synuclein (Angot et al., 2012; Desplats et al., 2009; Luk et al., 2012; Masuda-Suzukake et al., 2014).

In summary, our findings support the hypothesis that phagocytic cells are able to transport ingested cargo – including aggregated Aβ - from the periphery to the brain in a mouse model. Macrophages thus appear to play contradictory roles in Aβ deposition. On the one hand, they are predisposed to remove foreign substances and present antigen, but if they are unable to fully

degrade the material, they may inadvertently disseminate it to other parts of the body, including the brain. By clarifying the involvement of macrophages in the trafficking of proteopathic seeds, we hope to pinpoint key mechanisms that can be targeted to slow this process within the brain.

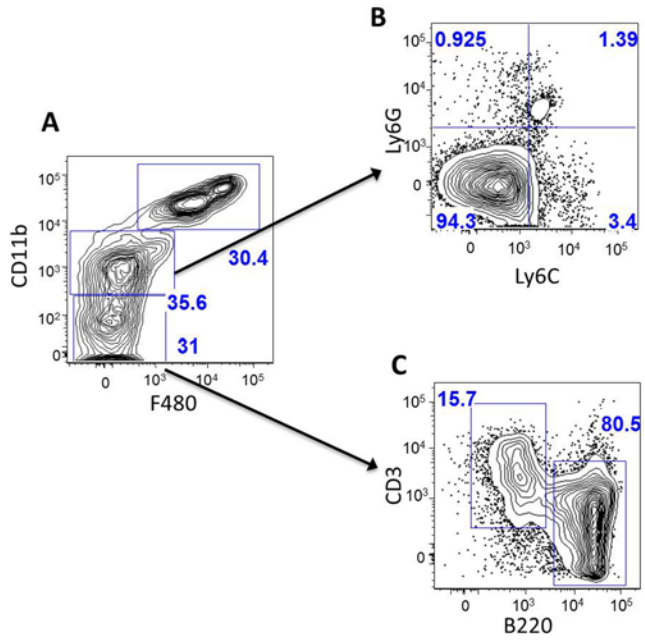

Figure 3.1.*Characterization of lavaged leukocytes by FACS analysis.* **A**. Whole lavage sample. The upper right gate (30.4%) represents CDllb-high & F4/80-high cells, which are large peritoneal macrophages (LPM). The middle gate (35.6%) represents CDllb-intermediate and F4/80-negative cells. This fraction of cells was further probed for expression of Ly6G and Ly6C (**B**). 94.3% of these cells were Ly6G-negative and Ly6C-negative, indicating that they are small peritoneal macrophages (SPM). The bottom gate of panel A denotes CDllb-negative and F4/80-negative cells. This fraction was further probed for markers of B-cells (B220) and T-cells (CD3) (**C**). 80.5% of these cells were B-cells and 15.7% were T-cells. Overall, this analysis indicates that the lavage cells consist of ~60% macrophages (LPM and SPM) and ~30% lymphocytes (most of which are B-cells).

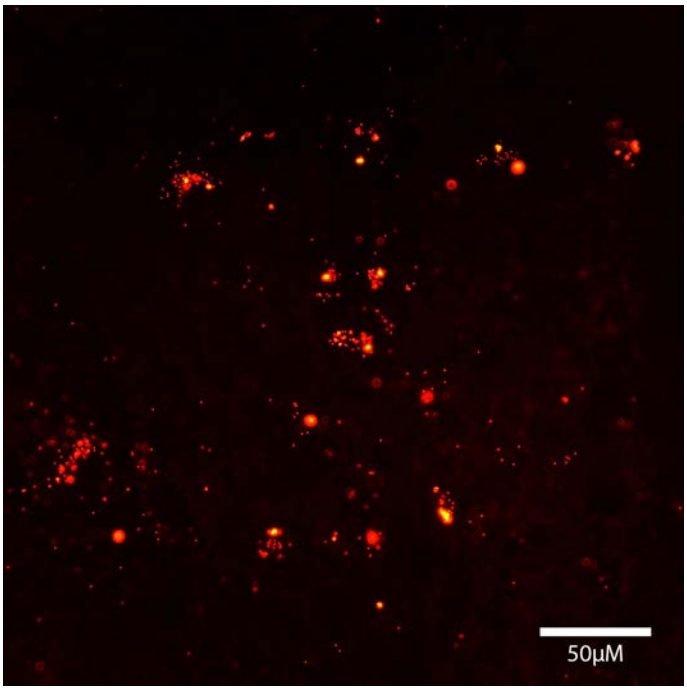

Figure 3.2: Fluorescent nanobeads (red) in the spleen of a host mouse 4 weeks after i.v. injection of bead-laden macrophages from a donor mouse.

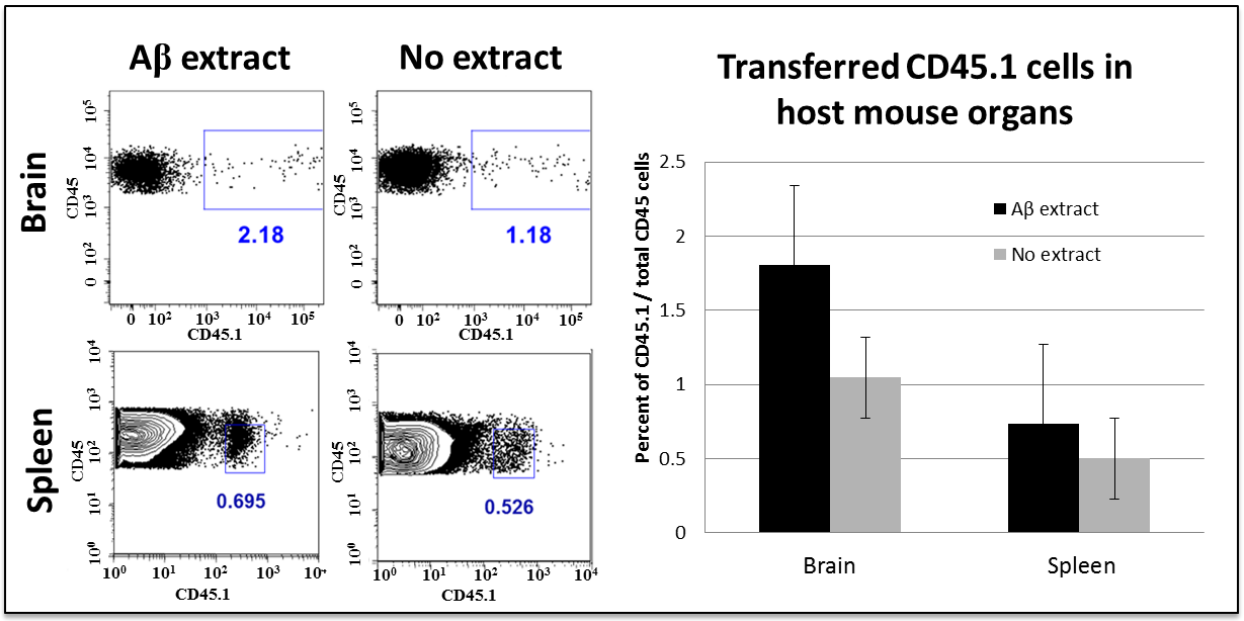

Figure 3.3. FACS analysis of intravenously transferred CD45.1 cells exposed to Aβ extract and control in the brain and spleen of a B6[CD45.2] host mouse. Cells were gated for CD45 immunoreactivity (all hematopoietic cells) and CD45.1 (exogenous donor cells). (Mice injected with Aβ-laden macrophages n=9 and control mice n=6) Exposure of the donor cells to Aβ-rich AD brain extract in the i.p. injectate did not significantly influence the relative quantity of transferred cells that entered the brain or spleen. (Plots on the left are representatives of the groups.)

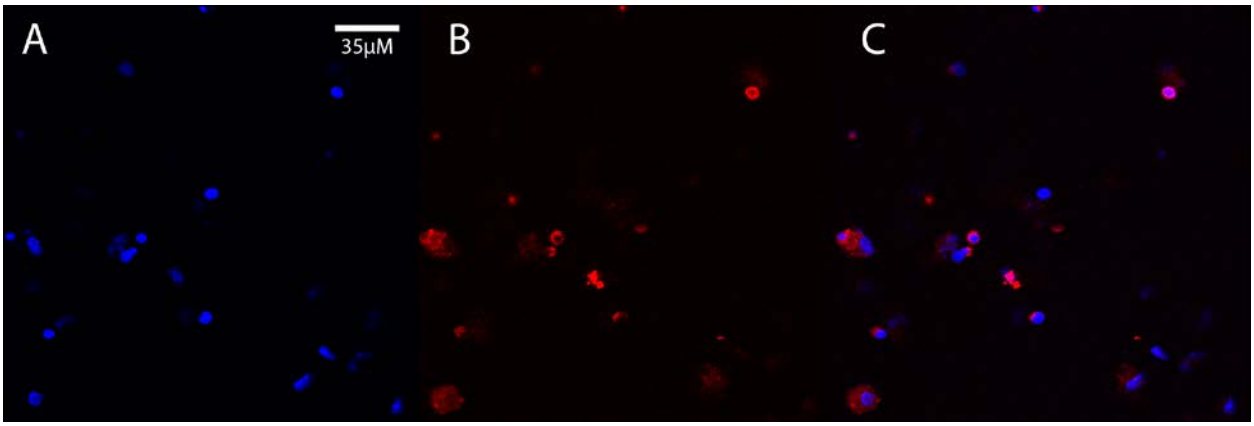

Figure 3.4. Macrophages harvested from the peritoneal cavity following i.p. injection of AD brain extract contain immunodetectable human Aβ. A) Cell nuclei stained with DAPI (blue); B) Aβ immunofluorescence (red, antibody 6E10); C) merged images.

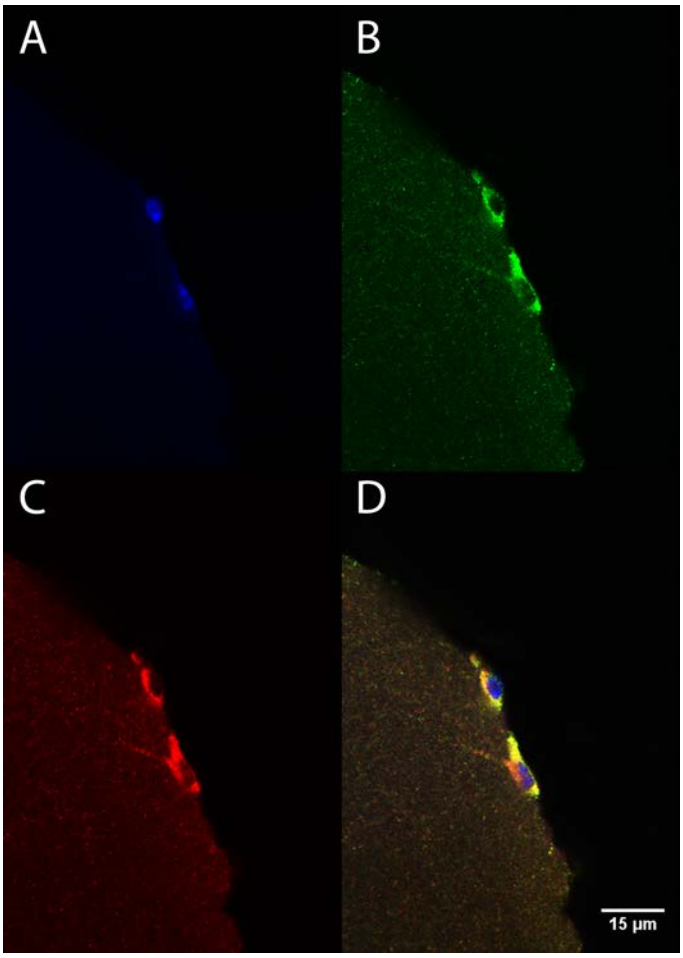

Figure 3.5. Exogenous macrophages from a CD45.1-expressing donor mouse in the superficial neocortex of a CD45.2-expressing host mouse. The cells were exposed to Aβ-rich AD brain extract in the peritoneal cavity of donor mice and injected intravenously into the host mice 24 hours prior to sacrifice. A) DAPI nuclear stain (blue); B) CD45.1 (green); C) Aβ (red); D) merged images.

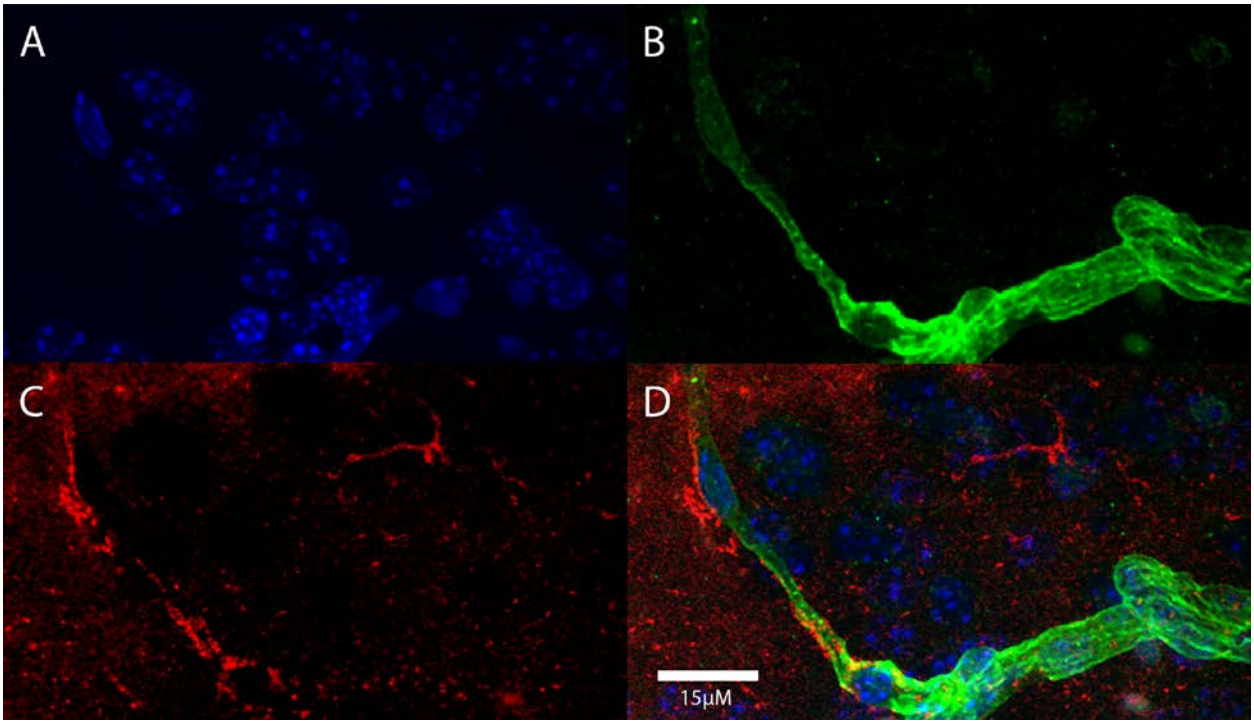

Figure 3.6. Aβ immunoreactivity (red) adjacent to a laminin-immunoreactive blood vessel (green) in the neocortex of a CD45.2-expressing host mouse 24 hours after an i.v. infusion of Aβ laden exogenous macrophages. In this case, the Aβ-immunoreactive cells were not demonstrably immunopositive for the donor antigen (CD45.1) (data not shown). A) DAPI nuclear stain (blue); B) laminin (green); C) Aβ (red); D) merged images.

3.6: Long-term effects of exogenous, Aβ seed-containing circulating monocytes on cerebral Aβ deposition in APP-transgenic mice.

The previous study demonstrated that macrophages are able to take up and transport material from the periphery to the brain through the vasculature. In this light, the findings support the hypothesis that peripheral macrophages could be a source of amyloidogenic Aβ seeds that traffic to the brain from the peritoneal cavity in an experimental seeding model (Eisele et al., 2010; Eisele et al., 2014). Here I have investigated the long term effects of exogenous, Aβ seed-laden circulating monocytes in the APP/PS1 transgenic mouse model.

3.6.1 Methods

Injection and lavage paradigm: Wild-type mice designated as donor mice received intraperitoneal (i.p.) injections of AD brain extract on day 0 and day 3, and sacrificed on day 4 as described above. Macrophages were collected by lavage, processed (1500 rpm for 5 min, red blood cell lysis), and counted. APP/PS1 transgenic host mice (2-month) were injected with donor macrophages either intracerebrally (i.c.) or intravenously (i.v.). Following a 7-month incubation period, mice were deeply anesthetized, perfused with cool (4-8^{o}C) PBS, and the brain and systemic organs collected for analysis.

Immunohistochemistry: The brain and systemic organs were cut in half: One half was fixed in 4% paraformaldehyde for 24 hours and then cryoprotected in 30% sucrose. The unfixed half was flash-frozen and stored at -80^{o}C for use in the enzyme-linked immunosorbent assay (ELISA). Fixed tissue was frozen, sectioned at 40 µm thickness on a cryostat, and mounted onto slides. The brain was stained with various antibodies to Aβ (monoclonal antibody 6E10 to Aβ1-16,

monoclonal antibody 4G8 to Aβ17-24, a monoclonal antibody to Aβ1-38; and polyclonal antibodies R361 to Aβ32-40, and R398 to Aβ33-42).

Plaque Counts: For each experimental group, multiple sections were evaluated for the amount of aggregated Aβ. To determine the quantity of plaques in the neocortex and hippocampus, a grid divided into 10 µm squares was placed over tissue sections that had been stained with various Aβ antibodies (see above). Plaques that intersected with axes on the grid were counted, the intersection counts converted to areas using stereological methods, and the area occupied by plaques was measured against the total area of the hippocampus and neocortex, respectively.

ELISA quantification of Aβ40 and Aβ42: For measurement of Aβ levels, fresh-frozen tissue was homogenized on ice with a Dounce homogenizer in cool RIPA lysis buffer. Lysate was cleared by centrifugation at 13,000 rpm for 10 min at 4°C. The supernatant was collected for measurement of soluble Aβ. For insoluble Aβ, the pellet was resuspended in 70% formic acid, sonicated for 10 min and neutralized with 1M Tris buffer (pH 7.4). Following a spin at 13,000 rpm for 10 minutes, the clear supernatant was collected as the buffer-insoluble extract. For both extracts, total Aβx-40 and Aβx-42 levels were measured by enzyme-linked immunosorbent assay (ELISA) using standardized kits (Millipore EZBrain).

3.6.2 Results

Cerebral Aβ42 immunoreactivity is decreased in mice infused with Aβ seed-laden macrophages

Mice intravenously injected with Aβ seed-laden macrophages showed decreased immunoreactivity to Aβ42 in the brain as assessed histologically (Figure 3.7). Specifically, Aβ42 deposits were significantly reduced in mice infused with macrophages that had ingested aggregated human Aβ, with a p-value of <0.05 in neocortex and in hippocampus (Figure 3.8).

There was no significant difference in areal density of plaques for Aβ1-16, Aβ1-38, or Aβ40 in either the neocortex or hippocampus.

Quantification of Aβ levels using ELISA

For both extracts, total Aβx-40 and Aβx-42 levels were measured by ELISA. Though there was a trend toward lower insoluble Aβ42 levels in mice intravenously injected with Aβ seed-laden macrophages compared to mice infused with control macrophages, the group difference was not statistically significant (Figure 3.9). No statistically significant difference was found for Aβx-40 levels (data not shown).

3.6.3 Discussion

Earlier studies found that long-term incubation of APP-transgenic mice that had received an intraperitoneal injection of Aβ-rich brain extract resulted in an increase in cerebral Aβ deposition (Eisele et al., 2009; Eisele et al., 2014). Based on these findings, and the observation of Aβ seed-laden macrophages in blood shortly after the i.p. infusion (Eisele et al., 2014), we hypothesized that long-term incubation of APP-transgenic mice infused with Aβ seed-laden macrophages would result in a similar increase in Aβ deposition. Surprisingly, following a 7-month incubation period the opposite effect was observed. Specifically, a decrease in Aβ42 was revealed by quantification of plaques in the mice that received exogenous Aβ seed-laden macrophages. These histopathological results, however, were not fully corroborated by ELISA quantification of Aβ42, which showed a small decrease in the amount of insoluble Aβ42 that was not statistically significant. The quantities of other Aβ fragments did not differ significantly in the experimental and control groups.

There are several potential explanations for this finding. It is possible that the groups did not differ at all, and that the significant lowering of Aβ42 histologically was a statistical anomaly. The fact that the ELISA results, though non-significant, were in the same direction as the immunohistochemical findings, however, suggests that the group difference is real. If so, we speculate that the decrease in Aβ42 could be the result of antigen-presentation by the macrophages. In other words, it is possible that the macrophages in the donor mice ingested the human Aβ and presented antigen fragments to other immune cells in the host mice, thereby generating an immune response that led to a reduction in cerebral Aβ load (Morgan, 2011; Schenk et al., 1999). A key difference between this study and the Eisele studies was the source of Aβ-rich extract. Our protocol used Alzheimer's disease brain extracts, whereas Eisele and colleagues used aged APP-transgenic mouse brain extracts from the same line of mice as the hosts (APP23 mice). The exposure to multiple human antigens could have elicited a greater immune response in our host mice, resulting in the generation of antibodies that partially cleared (or prevented aggregation of) Aβ. A significant sub-population of cells in the donor injectate were B-lymphocytes, which also could have contributed to an adaptive immune response in the host mice. Why Aβ42 appeared to be preferentially affected in this paradigm is unclear.

In light of our studies showing that macrophages can transport aggregated Aβ from the periphery to the brain (above), the long-term incubation experiment supports the view that macrophages play a dual role in the pathogenesis of AD-like proteopathy. On the one hand, they are able to take up and transport Aβ aggregates that could act as seeds for the deposition of Aβ in the brain. On the other hand, if an adaptive immune response is elicited by Aβ antigen-presentation, the macrophages can aid in the clearance of Aβ. It is important to establish the conditions under which these two opposing functions are optimized, as immune activation by antigen-presentation

could represent a cell-based approach to reducing Aβ deposition and thus possibly lowering the risk of Alzheimer's disease.

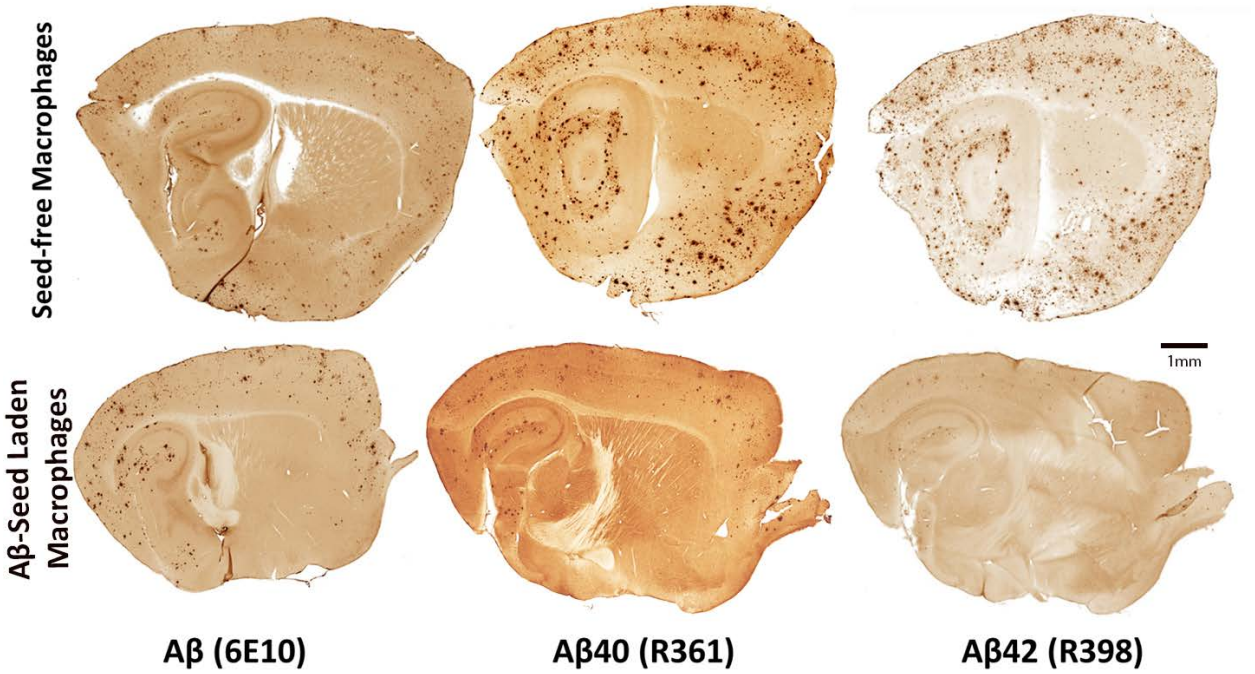

Figure 3.7. Aβ42 immunoreactivity is reduced in mice infused with Aβ seed-laden macrophages (bottom row) compared to mice infused with Aβ seed-free macrophages (top row). Shown are parasagittal sections immunostained for Aβ1-16 (antibody 6E10), Aβ40 (antibody R361), and Aβ42 (antibody R398). All sections were stained at the same time and under the same conditions.

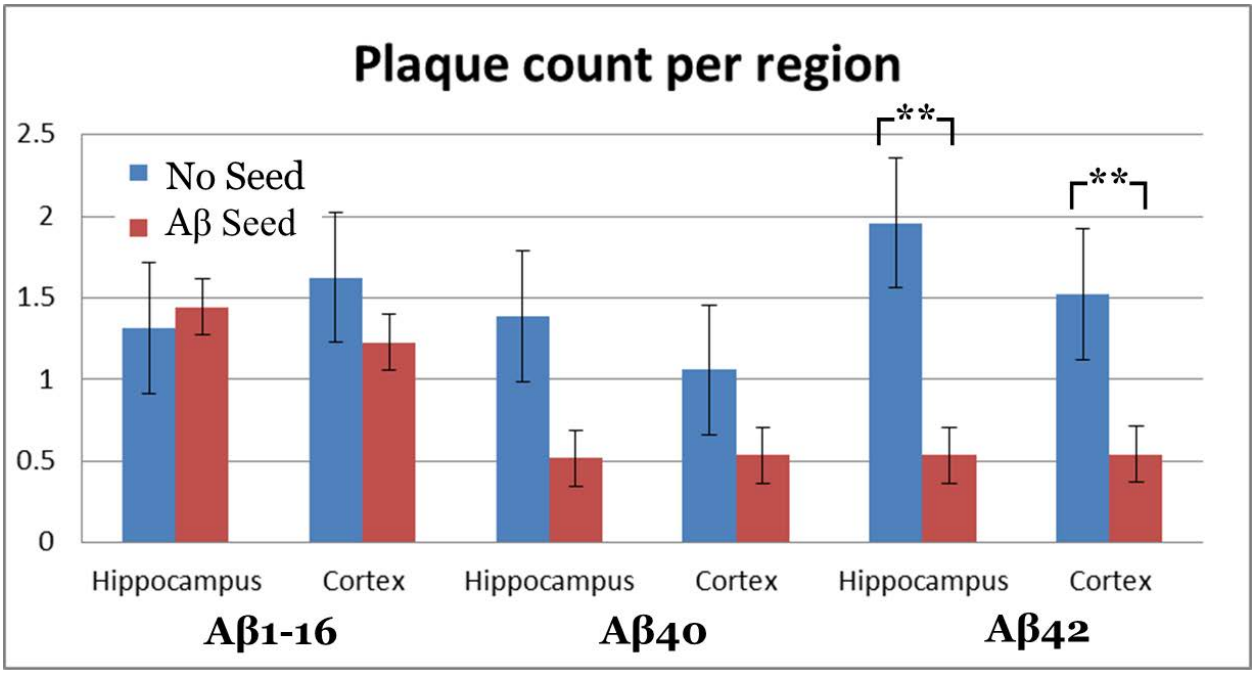

Figure 3.8. Quantification of Aβ-immunoreactive deposits in the hippocampus and neocortex of mice that received exogenous Aβ seed-laden macrophages or seed-free macrophages. Tissue sections were stained with antibodies to N-terminal Aβ (antibody 6E10) or to C-terminal Aβ (ending at amino acids 40 or 42) (** indicates p-value < .005).

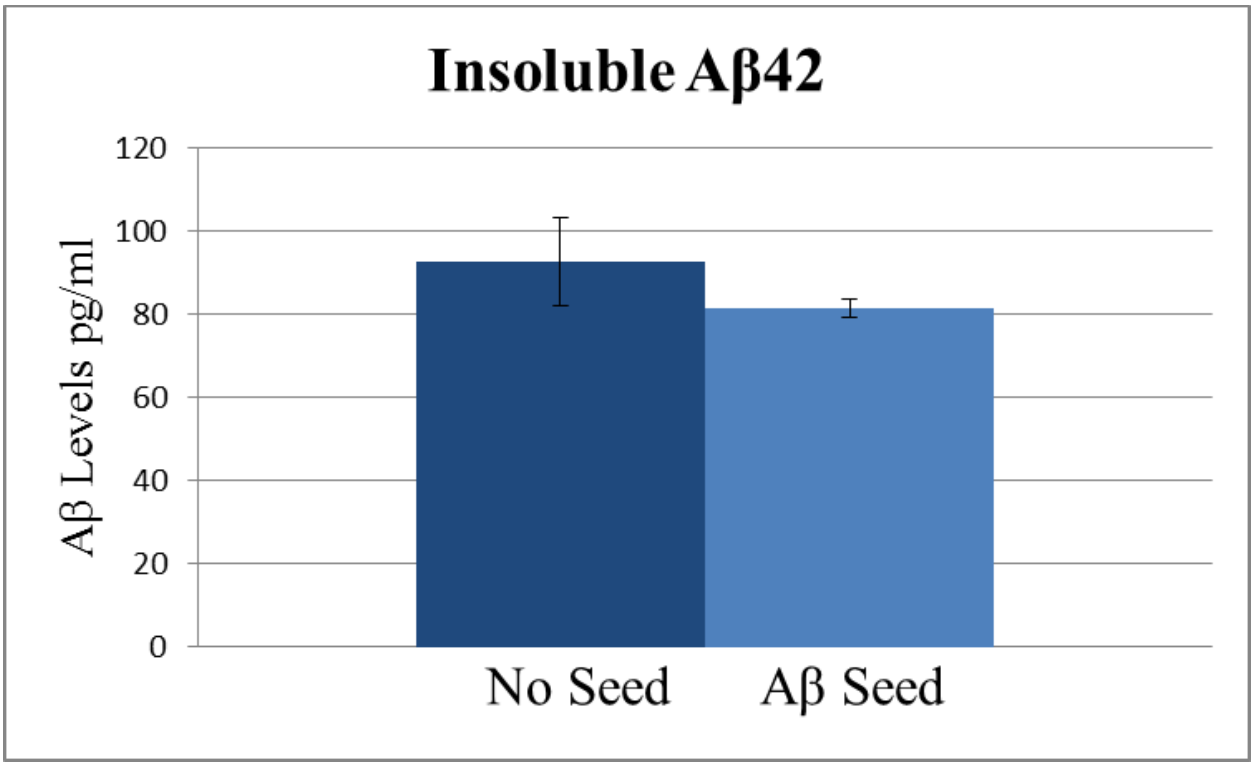

Figure 3.9. Insoluble Aβ42 levels as measured by ELISA were not significantly different between the groups of mice that received Aβ seed-bearing macrophages and those that received control macrophages (F= 2.2, p = 0.18).

Chapter 4

Seeding PIB-Positive Aβ Plaques and Cerebral Aβ Angiopathy in APP-Transgenic Mouse Models

4.1 Abstract

The misfolding and aggregation of the β-amyloid peptide (Aβ) is one of the earliest events in the pathogenesis of Alzheimer's disease. The induction and spread of these lesions involves a process of corruptive protein templating (seeding), similar to the molecular mechanism underlying prion disease. Experimentally, Aβ deposition can be induced in Aβ-precursor protein- (APP) transgenic mice by the intracerebral injection of dilute brain extracts that contain misfolded, aggregated Aβ seeds. Radiolabeled Pittsburgh compound B (PIB) binds with high affinity and specificity to cerebral Aβ deposits in patients with Alzheimer's disease. However, PIB high-affinity binding sites are very rare on Aβ deposits in APP-transgenic mice and aged nonhuman primates, even though these models express human-sequence Aβ. We asked whether high-affinity PIB binding sites can be generated on Aβ deposits that have been seeded by AD-brain extracts in APP-transgenic mice. Using autoradiographic assessment of ^{3}H-PIB binding to tissue sections, we found that high-affinity PIB binding is augmented in seeded deposits of Aβ in this model, suggesting that the exogenous seeds can influence the characteristics of the resulting protein aggregates *in vivo*. Surprisingly, seeding with brain extracts from aged APP-transgenic mice or aged nonhuman primates also increased PIB-positive deposits in host mice. In light of our finding that PIB-positivity increases somewhat in unseeded transgenic mice in very old age, this finding suggests that seeding acts by accelerating the maturation of the deposits in host mice. Establishing an understanding of the strain-like structural features of Aβ in Alzheimer's disease could provide new insights into the characteristics of proteopathic lesions that are specific to Alzheimer's disease.

4.2 Introduction

The hallmark pathology of Alzheimer's Disease is the presence of extracellular β-amyloid plaques and intracellular neurofibrillary tangles (Alzheimer, 1907; Holtzman et al., 2011; Khachaturian, 1985; Mirra et al., 1991). Radiolabeled Pittsburgh compound B (PIB; [N-methyl-^{11}C] 2-(4′-methylaminophenyl)-6-hydroxybenzothiazole) is a diagnostic imaging agent that binds with high affinity and stoichiometry to parenchymal β-amyloid deposits (Klunk et al., 2004; Nordberg, 2008; Rowe et al., 2007) and cerebral β-amyloid angiopathy (CAA) (Bacskai et al., 2007; Johnson et al., 2007) in humans. PIB high-affinity binding sites are very rare on Aβ deposits in APP-transgenic mice and aged nonhuman primates, even though these models express human-sequence Aβ (Klunk et al., 2005; Rosen et al., 2011). Variability in PIB binding affinity is seen even among patients with Alzheimer's disease (Ikonomovic et al., 2012; Rosen et al., 2010). In one documented instance, an Alzheimer's patient did not have significant high-affinity PIB binding despite having exceptionally large amounts of Aβ deposition in the brain (Rosen et al., 2010).

These studies suggest that PIB binds to a polymorphic molecular site on Aβ that is much more common in human AD cases than in experimental animals (Levine and Walker, 2010), but that in humans there may be a variety of Aβ conformational variants. Conformational differences in Aβ deposits have supported the hypothesis that aggregated Aβ, like prions, exists in the form of variant *strains* (Shewmaker et al., 2011; Tycko, 2014). Structural differences in Aβ have been observed in APP-transgenic mouse models and have been shown to conserve strain characteristics when seeded into other transgenic models (Fritschi et al., 2014; Heilbronner et al., 2013; Meyer-Luehmann et al., 2006). The lack of PIB binding in nonhuman primates and APP-transgenic mice lends support to the hypothesis that PIB recognizes a strain of Aβ that is

enriched in humans, a strain that is perhaps more pathogenic than putative strains in animal models. In this study, we tested the hypothesis that high-affinity PIB binding sites can be generated on Aβ deposits that have been seeded by AD-brain extracts in APP-transgenic mice.

4.3 Materials and Methods

4.3.1. Subjects. Eight male APPSwe/PSEN1 mice (2 months old at injection) were used as the seeding hosts, and two additional APPSwe/PSEN1 mice were included as unseeded aged controls (>24 months old). These APP/PS1 mice carry co-segregating transgenes for APP with the Swedish double mutation (K670N-M671L) along with presenilin-1 with a deletion in exon 9 under control of the prion promoter (Borchelt et al., 1997). Additionally, 24 3-3.5 month-old APP23 mice were used as seeding hosts and 5 APP23 mice were used as unseeded aged controls (>24 months)(Sturchler-Pierrat et al., 1997). The experimental procedures were carried out in accordance with US federal guidelines, and were approved by the Emory Institutional Animal Care and Use Committee.

Preparation of seeding tissue extracts. Neocortical tissue samples were obtained at autopsy from 3 AD cases and 2 age-matched controls (all samples were coded to ensure the anonymity of the donors). Animal extracts were obtained from two aged nonhuman primates: a 35-year old rhesus monkey and a 21-year old squirrel monkey, both with heavy cerebral Aβ deposition; from an aged (30 months) APP23 transgenic mouse; from an 11.5-month old APP/PS1 transgenic mouse (both transgenic models had substantial Aβ deposition); and from a wild-type control mouse. Ten percent (w/v) tissue extracts for injection were prepared by adding 9 parts PBS to tissue for Dounce homogenization. Once homogenized, tissue was probe-sonicated 10 × 1 s with a Fisher

Sonic Dismembrator 100 (setting 5) and spun at 3,000g for 5 min at 4°C. The supernatant was removed and stored at -80°C until use.

Injection and incubation of tissue extracts. APP/PS1 mice: 2.5 µl of clarified tissue supernatant were injected into the dorsal and ventrolateral hippocampus (2 injections per hemisphere, 4 injections total) of APP/PS1 mice. Mice were injected with AD tissue extracts in the right hemisphere, while the left hemisphere was injected either with control, non-demented human tissue extract, or with Aβ-rich brain extracts from aged nonhuman primates or APP/PS1 transgenic mice (aged 11.5 mo). All APP/PS1 host mice received injections at 2 months and were allowed to survive until 7 months of age. This time-course was used because we wanted to assess the emergence of seeding-induced pathology before significant endogenous (unseeded) transgene-induced pathology presented (which, in this model, usually begins at around 6 months of age (Borchelt et al., 1997)). All mice were sacrificed under deep (ketamine/xylazine) anesthesia by perfusion with PBS followed by phosphate-buffered 4% paraformaldehyde (pH 7.2). Following post-fixation for 24 hours, tissue blocks were embedded in paraffin and sectioned at 10 µM thickness on a rotary microtome.

APP23 mice: 2.5 µl of clarified brain extract (either Alzheimer's disease, non-demented, wild-type mouse or 30 month old APP23 mouse brain) were injected bilaterally into 3-4 month old APP23 mice. Mice were sacrificed after a 6-month incubation period under deep (ketamine/xylazine) anesthesia by perfusion with phosphate-buffered saline (PBS), pH 7.4. The right hemisphere was fresh-frozen and the left hemisphere was immersion-fixed in 4% paraformaldehyde for 24 hours. Fixed tissue blocks then were suspended in a buffered 30% sucrose solution, embedded in OCT, and sectioned at 15 µm thickness on a cryostat.

3H-PIB autoradiography

Slide-mounted sections were de-paraffinized in xylene, immersed sequentially in a descending series of ethanol concentrations (100%, 95%, 70%), and placed in PBS. Sections then were incubated for 24 hours in 1.0 nM or 4.0 nM ^{3}H-PIB (specific activity = 64 Ci/mmol) without or with 1.0 µM cold PIB as a control to block specific binding sites. After incubation, sections were rinsed 3 times in 50% ethanol for 10 minutes on ice and allowed to air-dry before direct exposure to either Hyperfilm ^{3}H (Amersham Biosciences, now GE Healthcare) or BAS-IP TR2025 E phosphorimaging screens (Fujifilm, Tokyo) in a cassette. For film, an 11 day exposure was followed by developing in Kodak D-19 developer, with the film dried and scanned for imaging. Phosphorimaging screens were exposed for 2 days and analyzed using a BAS5000 phosphorimager (Fujifilm, Tokyo). As positive controls, histopathologically confirmed AD tissue samples obtained at autopsy were fixed in buffered 4% paraformaldehyde, embedded in paraffin and further processed as described above.

Immunohistochemistry

The sections used for PIB binding or the adjacent sections were processed immunohistochemically using monoclonal antibody 6E10 to Aβ1-16 (1:10,000) or 4G8 to Aβ17-24 (1:5000). The mouse-on-mouse protocol (goat-anti-mouse FAB fragments during block and biotinylated goat anti-mouse FAB fragments as secondary) was used in staining murine material to eliminate background signal caused by the binding of secondary anti-mouse antibody to endogenous mouse tissue immunoglobulins and other factors. Vectastain Elite kits (Vector Laboratories, Burlingame, CA, USA) were used for avidin-biotin complex labeling of the primary antibody, with diaminobenzidine (DAB) as the chromogen. Positive control specimens

from AD cases with previously confirmed pathology were included in all immunostaining experiments.

4.4 Results

Some Aβ deposits in unseeded, aged APP-transgenic mice bind PIB with high affinity.

25-month old APP23 and APP/PS1 mouse brains were examined for high-affinity PIB binding. In APP/PS1 mice, PIB binding was low despite a high overall Aβ plaque burden. In both mouse models, PIB bound primarily to Aβ plaques in the neocortex and to a lesser extent to CAA in the cortex and thalamus (Figure 4.1).

Seeding with Alzheimer's disease or aged APP-transgenic mouse brain extract elicits PIB binding

Brains of 7-8-month old host mice seeded with brain extract from Alzheimer's disease patients or from aged APP-transgenic mice bind low nanomolar ^{3}H-PIB. PIB bound mostly to deposits surrounding blood vessels in the seeded mice. In APP23 mice, the majority of CAA-positive PIB was in the thalamus (Figure 4.2). The APP/PS1 mice had a more varied pattern of PIB signal, although, as in the APP23 mice, CAA showed the most prominent binding. Age-matched APP23 (Figure 4.2) and APP/PS1 (data not shown) control hosts that received non-demented or wild-type brain extract did not show PIB binding in brain.

*Seeding with Aβ-rich brain extracts from aged nonhuman primates also elicits high-affinity PIB binding*APP/PS1 host mice that received intracerebral nonhuman primate tissue extract injections also showed high-affinity binding of ^{3}H-PIB to some Aβ deposits. The pattern of PIB binding was consistent with predominant binding to CAA; however, a faint band of PIB binding was seen along the hippocampal fissure, corpus callosum and fornix, which corresponded to Aβ staining with antibody 6E10 (Figure 4.3).

4.5 Discussion

The process of Aβ seeding is thought to follow the prion propagation paradigm, i.e., a misfolded protein acts as a corruptive template, inducing like proteins to misfold, aggregate, and form oligomeric and fibrillar multimers (Jucker and Walker, 2011; Walker et al., 2013). In this study, we introduced β-amyloid seeds from different donors to test the hypothesis that the PIB-binding characteristics of Aβ aggregates can be propagated from specific donors to host mice (Fritschi et al., 2014). We used PIB binding as an indirect indicator of variant strains of Aβ. At low nanomolar concentrations, PIB binds to human Aβ with high affinity, whereas PIB only binds in low amounts to Aβ in aged monkeys and APP-transgenic mice (Rosen et al., 2010; Rosen et al., 2011). The present findings, however, demonstrate that, regardless of the nonhuman donor, seeding with Aβ-rich brain extract results in a degree of PIB binding in the hosts that is greater than binding in the original donors. We also show that aggregated Aβ in the seeding extract is necessary to induce PIB binding. Brain extracts from non-demented, aged humans or from wild-type mice did not seed Aβ deposition or otherwise induce PIB binding.

In addition to the induction of PIB binding in seeded hosts, we also found, in two different mouse models, that PIB binds with high affinity to some Aβ lesions in the brains of very old, unseeded APP-transgenic mice (>24 months). Thus, it appears that by seeding deposition in younger mice, we are accelerating the timecourse of Aβ aggregation, thereby causing larger amounts of deposition at an earlier age. Accordingly, one possible explanation for our findings is that seeding advances Aβ deposition such that the lesions in younger mice resemble those that occur spontaneously in very old mice, thereby resulting in PIB binding regardless of whether Aβ in the donor tissue bound PIB. This hypothesis is supported by the recent finding that oligothiophene binding to Aβ deposits evolves as transgenic mice age (Nystrom et al., 2013). To

further address this issue, seeding with different donor extracts could be undertaken in a host animal that develops deposits that never bind PIB, even at an advanced age. This would enable us to determine if PIB binding is induced due to a seeded conformational change, or whether it is related solely to the age of the Aβ deposits.

It is also interesting that seeding in both APP-transgenic mouse models resulted in a large amount of Aβ deposition in the walls of cerebral blood vessels (CAA). While both aged models spontaneously develop some degree of CAA, the majority of deposited Aβ is parenchymal, indicating that seeding induces a shift in the histological pattern of Aβ deposition. CAA is most pronounced in the thalamus of seeded APP23 mice, and in the superficial cortical layers of APP/PS1 mice. Since both models received hippocampal injections, this difference in the primary location of vascular deposition remains unexplained, but could reflect a difference in the transport or distribution of the seeds in the two models. Perhaps a more likely explanation is that the pattern of CAA reflects differences in transgene expression patterns and the inherent tendency toward the pattern in two mouse models as they normally age. For example, thalamic CAA is fairly common in aged, unseeded APP23 mice (Sturchler-Pierrat et al., 1997).

This study sheds light on the ability of seeding to influence the characteristics of Aβ in APP-transgenic mouse models, hastening the onset and possibly the evolution of Aβ deposition. The PIB-binding data, in conjunction with studies of oligothiophene binding (Fritschi et al., 2014; Heilbronner et al., 2013; Nystrom et al., 2013), suggest that seeding also affects the conformation of Aβ molecules in the lesions. The existence of conformational strains of Aβ could account for disparities in Aβ pathology. Many other mammalian species deposit aggregated Aβ in the brain with age, yet they do not exhibit most other disease characteristics of Alzheimer's disease (Heuer et al., 2012; Rosen et al., 2011). This is also true of some humans;

many instances have been reported of aged humans with a heavy Aβ burden in the absence of significant memory deficits (Bouras et al., 1994; Braak and Braak, 1997; Wolf et al., 1999). The high affinity and stoichiometric preference of PIB for human Aβ is a useful tool for examining these disparities (Ikonomovic et al., 2012; Rosen et al., 2010). Further investigation is needed to elucidate the pathogenicity of Aβ in humans and the existence and functional importance of Aβ strains.

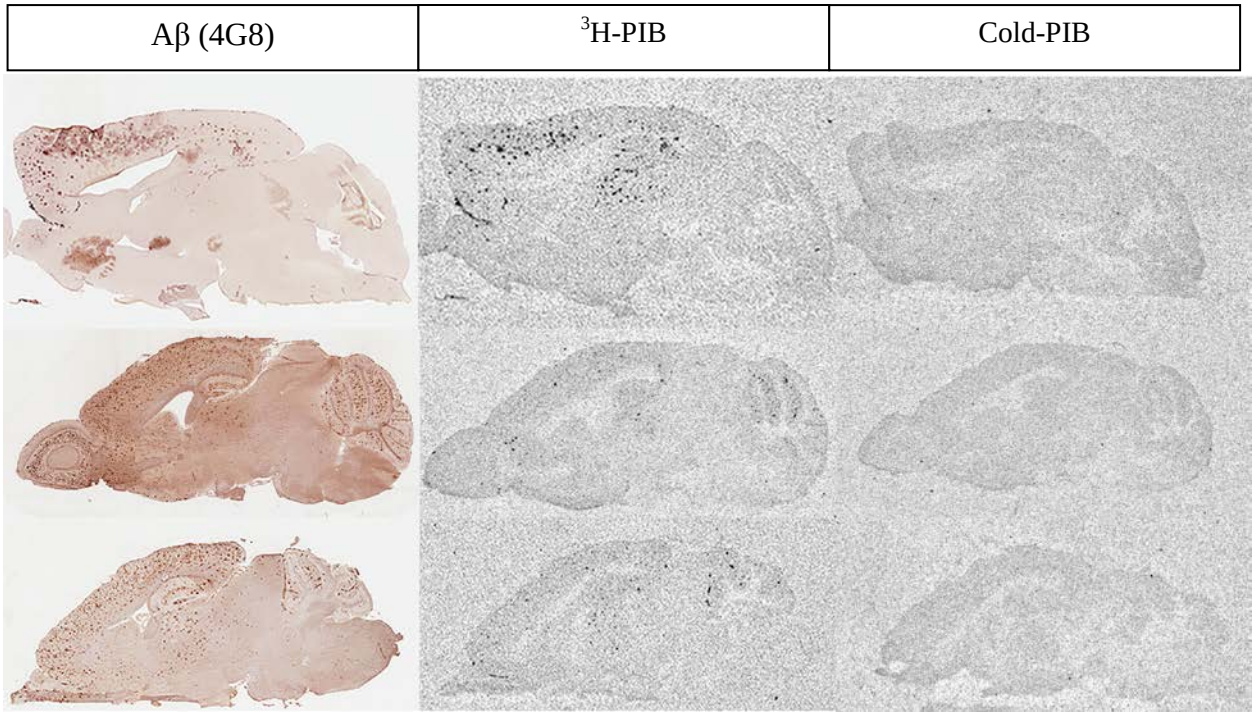

Figure 4.1. Some Aβ deposits in aged, unseeded APP-transgenic mice bind PIB. Top row: 25-month old APP23 mouse. Middle and bottom rows: 24- and 25-month old APP/PS1 mice. ^{3}H-PIB autoradiography at 1.0nM concentration. Right-hand column: Cold PIB (1μM) competition control on adjacent sections.

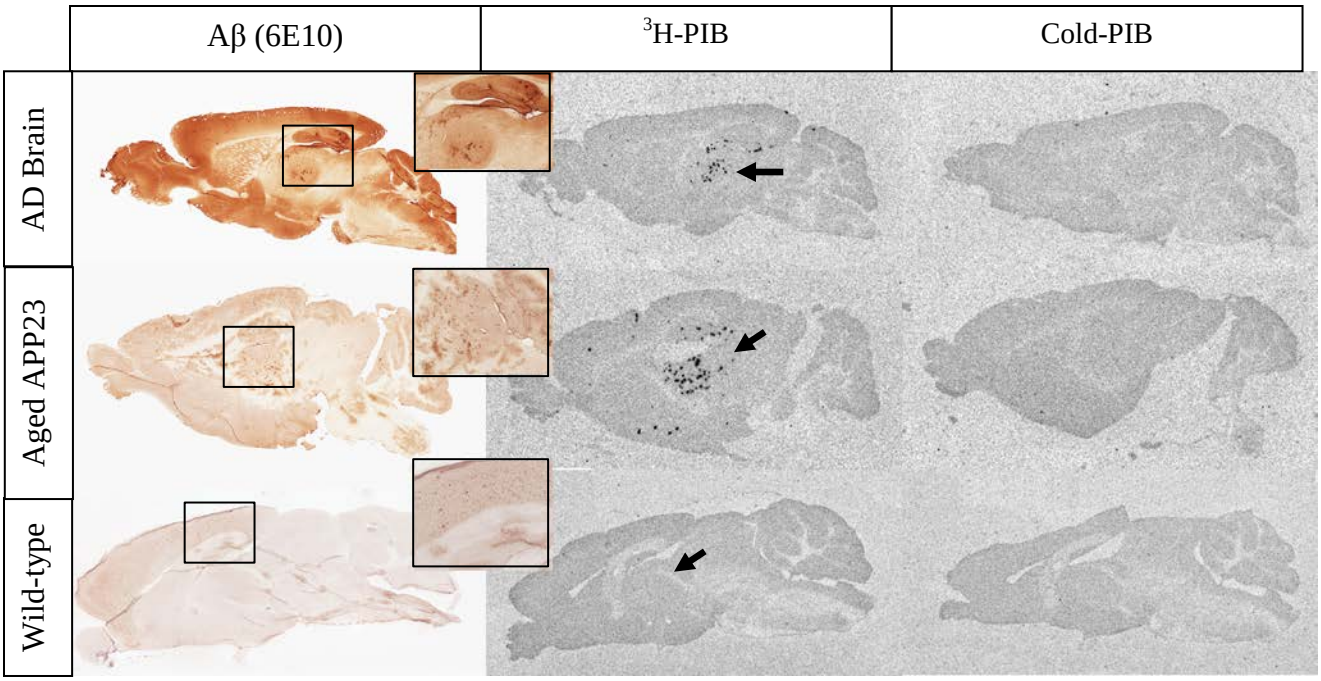

Figure 4.2. Top and middle rows: In APPtg host mice seeded with brain extracts from AD patients (top) or APP23 mice (middle), PIB binds primarily to CAA in the thalamus (arrows in the middle column indicate thalamus). Bottom row: APP23 control extract-seeded mice show little or no PIB binding. ^{3}H-PIB autoradiography at a 1.0 nM concentration. Right-hand column: Cold PIB competition control on adjacent sections.

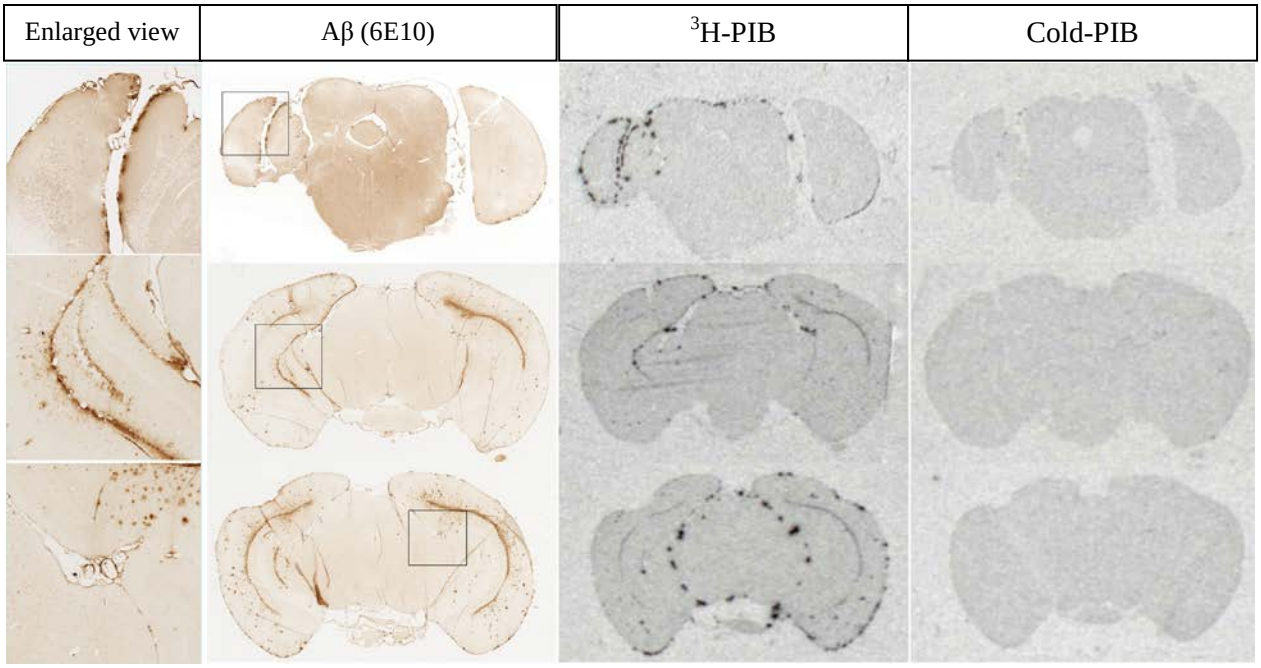

Figure 4.3. PIB binding in AD-seeded APP/PS1 mice. ^{3}H-PIB autoradiography at a 4.0nM concentration. Right-hand column: Cold PIB competition control on adjacent sections. Mice received bilateral injections of Aβ rich brain extract (left non-human primate; right Alzheimer's disease). Much of the binding is in superficial regions and CAA; note also some binding in the corpus callosum.

Chapter 5

Discussion and Future Directions

5.1. Similarity of prions and Aβ aggregates

The concept that a protein could be an infectious agent was inconceivable 50 years ago. Kuru, scrapie and other prion diseases had been researched for decades before it was discovered that the infectious agent was not a virus, as had been thought, but an abnormally folded protein, now known as the prion protein (PrP). The protein misfolding model is now believed to represent the mode of pathogenicity for myriad diseases, but many important issues remain unresolved. The thesis work described herein has elucidated some of the ways in which the characteristics of one proteinaceous agent, aggregated Aβ, resemble those of prions.

The prion frontier has been plagued with controversy, but at the same time has yielded surprising new discoveries. The infectivity of prions has sparked anxiety about the potential dangers of prion diseases, particularly since the advent of bovine spongiform encephalopathy and its transmission to humans (Prusiner and Hsiao, 1994; Prusiner, 1998). The concern was fueled further when standard methods used to neutralize viruses on medical instruments were found to be ineffective against prions (Delrue et al., 2012; Gordon, 1946; Pattison, 1965; Pattison, 1972). Like prions, Aβ seeds have been found to resist other forms of neutralization such as boiling or drying (Eisele et al., 2009; Meyer-Luehmann et al., 2006). In a collaborative study with colleagues at the University of Tübingen, I investigated the ability of formaldehyde to interfere with the transmission of Aβ seeds (Chapter 2). We found that formaldehyde-fixed samples from human Alzheimer's disease brains and APP-transgenic mouse brains were able to seed pathology in an APP-transgenic mouse model. Aβ seeds remained active even after the tissue had been in fixative for two years, albeit with reduced potency. The reduced efficacy of the seed after exposure to harsh conditions was also seen in previous studies in which Aβ-rich

brain extract was boiled briefly prior to infusion into transgenic mice (Meyer-Luehmann et al., 2006). The remarkable durability of Aβ seeds in formaldehyde provides compelling new evidence that the inductive agents, like prions, are not conventional pathogens such as bacteria or viruses.

Another unexpected feature of prions is that the proteinaceous particles exist in the form of structurally and functionally different *strains* (Collins et al., 2004). Each strain manifests its properties in causing a distinct disease with characteristic clinical and pathological features (Caughey et al., 2009; Collinge and Clarke, 2007; Collins et al., 2004; Prusiner, 1998). One method of probing the molecular architecture that defines strains is through the use of luminescent conjugated oligothiophenes (LCOs), which report on subtle structural differences among protein molecules (Klingstedt et al., 2011). Previous experiments showed that LCOs can be used to differentiate strain-like features of Aβ deposits in transgenic mouse models (Heilbronner et al., 2013; Klingstedt et al., 2011; Nystrom et al., 2013). My studies with the Tübingen group showed that strain-like features detected by LCOs exist between two different APP-transgenic mouse models, and that these traits can be maintained even after the seeding extract had been fixed with formaldehyde (Fritschi et al., 2014). Strain-like differences among models may explain much of the phenotypic variability from one transgenic mouse model to the next. Such variability is seen in Aβ deposits in the parenchyma versus cerebral β-amyloid angiopathy, in the morphology of the lesions, in Aβ seeding efficacy, in behavioral differences among models, and possibly in the effects of the pathogenic process on the cells of the brain. Strain-like variants and their properties also may help to explain the human specificity of Alzheimer's disease. For example, it is conceivable that differences in the molecular

architecture of Aβ and its multimers influence the toxicity of the molecules in humans, and between humans and nonhuman species (see below). In my studies of Aβ deposits that were induced by fixed brain extracts, the formaldehyde may have 'fixed' the conformational features of Aβ in each model, thereby enhancing (or preserving) the induction of a specific conformation of Aβ in the seeded APP-transgenic host mice. A practical benefit of this finding is that strain-like properties of Aβ can still be analyzed in archived material after years in fixative.

There is growing evidence that the *in vivo* imaging agent Pittsburgh compound B (PIB) differentially binds to Aβ deposits in different cases of AD (Rosen et al., 2010), and in humans versus nonhuman primates (Rosen et al., 2011). Normally, PIB does not appreciably bind with high affinity to Aβ deposits in nonhuman primates (Rosen et al., 2011) or APP-transgenic mice (Klunk et al., 2005). In my studies, I attempted to elicit high-affinity PIB binding in transgenic mouse models by seeding the mice with brain extracts from AD patients. The findings generally support the idea that there are different molecular conformations of Aβ, in that PIB only bound to Aβ when seeded, or in some deposits in very old mice, indicative of conformational variability in Aβ. Unexpectedly, however, some degree of PIB binding was elicited even in host mice that were seeded with Aβ-rich brain extracts from aged nonhuman primates or from aged APP-transgenic mice (Chapter 4). These findings support a previous observation that oligothiophene spectra in senile plaques change as transgenic mice grow older, and suggest that some structural characteristics of Aβ deposits evolve as the lesions age. Whether similar changes occur in deposits of prion protein with age is unknown.

One of the remarkable features of prion disease – and early evidence that the agent of transmission was not a conventional pathogen – is that signs of an active immune response are absent (Prusiner, 1982; Prusiner, 1998). However, microglial cells and astrocytes in the brain do become activated in prion disease (Bradford and Mabbott, 2012) and in AD (Britschgi and Wyss-Coray, 2007). In addition, though typically involved in protecting the body from microbes and viruses, immune cells also are able to propagate pathology in prion disease (Aguzzi et al., 2013). Prion rods have been shown to be transported to lymphatic organs by dendritic cells (Johnson et al., 2010), and prions are thought to reach the brain via lymphoreticular cells and peripheral nerves (Aguzzi et al., 2013). In the case of Aβ, there had been indirect evidence that macrophage-type cells are involved in the uptake and dissemination of Aβ seeds (Eisele et al., 2010; Eisele et al., 2014). My study supports the view that, at least in an experimental model, macrophages can phagocytose Aβ and transport it to the brain intact. Surprisingly however, in my model, exogenous, Aβ-containing macrophages did not increase Aβ load in host mice, but rather may have caused a slight decrease in Aβ42 levels. The reason for this decrease is uncertain at present, but suggests that the human brain extract might stimulate Aβ-antigen-presentation by immune cells in our mouse model, whereas mouse brain extracts (Eisele et al., 2010; Eisele et al., 2014) do not. This explanation is further supported by the considerable amount of B-cells in the lavage fraction. Both macrophages and B-cells are professional antigen-presenting cells (Harvey et al., 2007). If they recognize the human brain material as foreign and present Aβ antigen (especially Aβ42) to the host, the resulting adaptive immune response could explain the decrease in immunoreactive Aβ after long-term incubation (below). Further studies are needed to address this issue, but overall, these studies support the concept that Aβ seeds, like prions, are quite resilient, and, though the immune cells involved appear to differ,

components of the immune system can participate in the processing and spread of seeds within the body (below).

5.2 Inflammation and Alzheimer's disease

The immune system is the body's primary defense against harmful agents, and it is no surprise that the study of this system is important as it relates to Alzheimer's disease (Britschgi and Wyss-Coray, 2007). Taking an objective look at the cells most closely involved in the pathogenesis of Alzheimer's disease, phagocytic cells are an obvious choice. Clearance of pathogenic agents and debris is typically facilitated by various types of macrophages in the periphery, and by the resident macrophages in the CNS, the microglia. The notion that macrophages may act as double-edged swords that can both protect the organism and inflict damage is not novel. Indeed, in disease states where there is chronic inflammation and/or constant presence of pathogens, macrophages can do more harm than good. Circulating monocytes have been shown to ingest and transport viruses, thereby disseminating the disease to lymphatic organs (Ferreira et al., 2010). Circulating monocytes are also capable of ingesting and transporting prions (Johnson et al., 2010), and under certain conditions have been shown to enter the CNS (Aucouturier et al., 2001).

When the peripheral injection of Aβ seeds was found to induce the deposition of Aβ in the brains of APP-transgenic mice, the possibility of circulating monocytes as the mechanism of transport became apparent (Eisele et al., 2010; Eisele et al., 2014). The preponderance of cerebrovascular amyloidosis in the host mice further suggested a vascular route of entry into the brain (Eisele et al., 2010). My thesis work has shown that peripheral macrophages are able to ingest and

transport Aβ seeds to the brain. One day after infusion of cargo-laden macrophages from donor mice, the Aβ was found both within the exogenous macrophages in which they were transported and, in some instances, in mostly perivascular macrophages that appeared to be independent of the exogenous macrophages that originally carried the seeds (Chapter 3). The presence of human Aβ in perivascular macrophages supports the hypothesis that the CAA seen by Eisele et al. (2010; 2014) following systemic infusion of seeds results from the vascular transport of seeds to the brain. At this point, it is unknown why the perivascular macrophages in my experiments lacked immunoreactivity to the antigenic marker for donor cells. Since the seeds infused into the host mice were within cells at the time of infusion, I speculate that the seeds may have been transferred from donor cells to host cells. Alternatively, since most, but not all, cells were demonstrably alive at the time of infusion, it is conceivable that seeds were released from nonliving (or even viable) cells, and reached the brain in a free state. It is unlikely however, that such seeds would cross the blood-brain barrier, and in any case the quantity of such seeds would be extremely small. Furthermore, an earlier study indicated that direct i.v. infusion of seed-bearing brain extract was not an effective way to seed cerebral amyloidosis (Eisele et al., 2009).

Compared to the mostly vascular localization of (apparently) secondary macrophages containing Aβ, exogenous (primary) cells containing the Aβ seeds were mainly located in superficial layers of the neocortex 24 hours after infusion. This localization aligns well with the anatomical pattern of PIB binding that was seen following the intracerebral injection of various seeding extracts into APP/PS1 transgenic mice. In those studies, the APP/PS1 mice were seeded with brain extracts from Alzheimer's disease patients or aged monkeys. After a long incubation period, the resulting Aβ deposition and PIB binding were largely colocalized in the superficial

layers of the cortex and around Aβ-containing blood vessels. Interestingly, this pattern of strong superficial PIB binding was not apparent in seeded APP23 mice, although PIB-positive CAA was prominent, particularly in the thalamus.

The other side of the double-edged sword that is macrophages became apparent in the long-term incubation studies. Here, we unexpectedly saw a reduction in the amount of Aβ42 in mice that received a single, i.v. infusion of Aβ seed-laden macrophages 7 months earlier. One obvious explanation for this finding is simply that, in this paradigm, the immune system did its job; injecting the donor mice with human Alzheimer's disease brain extract may have elicited an immune response that was absent in host mice that received brain extracts from the same line of transgenic mice (Eisele et al., 2010; Eisele et al., 2014). Accordingly, I speculate that the donor macrophages and/or B-cells that were transferred into the APP-transgenic mice, reacting to human antigens, presented Aβ antigen to the host immune system, essentially immunizing the host mice against human Aβ. Once this immune response was initiated in the host mice, endogenous phagocytic cells were alerted to Aβ42 and aided in its clearance. Since I expected from earlier findings that Aβ deposition would be increased (Eisele et al., 2010; Eisele et al., 2014) and that an immune response would not be elicited (Meyer-Luehmann et al., 2006), serum was not collected for analysis of antibody levels. Therefore, the antigen-presentation hypothesis remains to be formally tested. In any case, in this series of studies taken as a whole, we are confronted with the complexity of the immune system and the inherent duality of the response that is provoked by disease. If immune function is to become a therapeutic target for Alzheimer's disease, further investigation is needed to determine exactly when and how aspects of this system can be beneficially manipulated.

5.3 Future Directions

To fully characterize putative proteopathic strains of Aβ in Alzheimer's disease, conformation-sensitive molecules such as LCOs are a powerful tool (Klingstedt et al., 2011; Heilbronner et al., 2013). Using these compounds to examine various cases of Alzheimer's disease with distinct clinical histories can potentially elucidate the existence of structural Aβ strains, and how they are linked to the course and pathology of the disease. Similarly, further investigation of PIB binding may shed light on the value of selective ligands in defining the pathogenic nature of multimeric Aβ and other proteins. Studies using the PIB-binding fraction of Aβ from AD brains (Matveev et al., 2014) to seed the deposition of Aβ are currently underway. To expand on my thesis work, finding an APP-transgenic mouse model that does not bind PIB, even in advanced age, would be a useful paradigm for testing the effects of Aβ seeds on the induction of PIB binding using Aβ-rich brain extract. Furthermore, numerous Aβ-binding compounds have been, or are being, developed as diagnostic imaging agents, and virtually nothing is known about whether these compounds, like PIB, can detect molecular structural features that could inform us about the natural variability of abnormal protein deposits in AD. As mentioned above, one implication of our work on fixation and Aβ is that archived, fixed tissue can be used to interrogate the strain-like properties of Aβ even after the tissue has spent extended periods in formaldehyde.

Inflammation in Alzheimer's disease is a broad topic about which much remains to be discovered. Fully understanding the role of macrophages in the proteopathic seeding model will require more research, focusing in particular on the possible transfer of seeds between macrophages, the functionality and fate of macrophages within the brain, and their possible role in antigen presentation and how this might differ depending on the source of the brain extract. In

addition, we employed a single infusion of exogenous macrophages, and the effects of multiple infusions or chronic exposure to seed-laden macrophages need to be determined. When more effective Aβ seeds can be generated from synthetic peptide *in vitro*, these will provide a more robust system with which to clarify the pathobiology of Aβ strains. Finally, it will be informative to establish how systemic inflammation influences that functionality of immune cells as they interact with the brain, and whether this might influence the processing of Aβ by these cells.

Alzheimer's disease results from progressive cellular dysfunction that ultimately culminates in memory decline, behavioral impairment and death. At a molecular level, the disease process of seeded protein aggregation appears to have more in common with prion diseases than had been previously thought. Additionally, the complex involvement of inflammation, which has both detrimental and beneficial consequences, further complicates understanding the disease process. Gaining a fuller comprehension of the role of Aβ and the immune system in pathogenesis is necessary to develop an effective therapy for Alzheimer's disease. In the context of previous studies, this thesis work demonstrates the multidimensional variability of aggregated Aβ, and highlights the challenges faced in eliminating these pathogenic proteins once they have misfolded and aggregated. Truly effective therapeutic intervention in the disease process is most likely to result from early treatment that prevents the formation or abrogates the pathogenicity of abnormally aggregated proteins.

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