DEVELOPMENTAL LEAD EXPOSURE AND THE EXACERBATION OF ALZHEIMER'S PATHOLOGY: AN IMMUNOLOGICAL ANALYSIS

by

Annalise Noelle vonderEmbse

July, 2014

Director of Thesis/Dissertation: Dr. Jamie DeWitt

Major Department: Biomedical Sciences, Pharmacology and Toxicology

Early neuroimmune dysfunction may play a pivotal role in the etiopathology of Alzheimer's disease (AD), as it is hypothesized that many late-stage adult diseases have an early-life basis. Here we explore whether exposure to a known neuroimmunotoxicant during a period of developmental susceptibility in the central nervous system (CNS) parenchyma exacerbates the pathologies in an AD prone triple transgenic mouse model (3xTgAD). This "double-hit" research design modeled the high variability in AD due to detrimental exogenous influences, rather than the minority of AD cases that have a well-defined genetic origin and regular neurodegenerative progression. Triple transgenic mice were treated by gavage with lead acetate from postnatal day 5-15, a critical developmental window for microglia, immune cells of the CNS that are thought to play a major role in shaping the CNS. Microglial phenotype and colocalization with amyloid-β, the protein associated with AD senile plaques, were analyzed to detect changes in pathological severity. Our data indicate that early exposure to a neurotoxicant increases the number of activated microglia with age, which we hypothesize is due to either degradation of homeostatic inhibitory signaling pathways associated with early onset synaptic degeneration or over-burdened microglial phagocytic load. Furthermore, microglial activation states differed between genders and fluctuated with age, suggesting a sex-specific component to AD pathology

and potential correlation of neurodegenerative diseases with hormone receptors in the sexual differentiation of the developing brain.

DEVELOPMENTAL LEAD EXPOSURE AND THE EXACERBATION OF ALZHEIMER'S PATHOLOGY: AN IMMUNOLOGICAL ANALYSIS

A Thesis/Dissertation

Presented to the Faculty of the Department of Pharmacology and Toxicology

East Carolina University

Brody School of Medicine

In Partial Fulfillment of the Requirements for the Degree

Master's in Biomedical Sciences

by

Annalise Noelle vonderEmbse

July, 2014

DEVELOPMENTAL LEAD EXPOSURE AND THE EXACERBATION OF ALZHEIMER'S PATHOLOGY: AN IMMUNOLOGICAL ANALYSIS

by

Annalise Noelle vonderEmbse

APPROVED BY:

DIRECTOR OF
DISSERTATION/THESIS: _____
(Jamie C. DeWitt, PhD)

COMMITTEE MEMBER: _____
(Jamie C. DeWitt, PhD)

COMMITTEE MEMBER: _____
(David A. Taylor, PhD)

COMMITTEE MEMBER: _____
(Mark D. Mannie, PhD)

COMMITTEE MEMBER: _____
(Qun Lu, MS, PhD)

DIRECTOR OF THE MASTER'S OF
BIOMEDICAL SCIENCES PROGRAM: _____
(Richard A. Franklin, MS, PhD)

DEAN OF THE
GRADUATE SCHOOL: _____
(Paul J. Gemperline, PhD)

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my thesis advisor Dr. Jamie DeWitt, who was vital to my growth as a student and a scientist. She has gone above and beyond to support me, and challenge me to be a better researcher than what I ever thought possible. It would in no way be an overstatement to say her immeasurable patience, encouragement, and confidence in me is the primary reason for my success in my Master's training. Thank you, thank you, thank you, Jamie.

I would also like to thank my amazing lab mates Jason Franklin, Qing Hu, Dakota Johnson, and Corey Boles, who've gone out of their way to assist whenever possible, give an inspiring nudge, or help decapitate mice because of my complete inability to do so. I also need to acknowledge my wonderful family, as well as my G-Vegas subfamily, Nate, Meg, and Squid, who provided more support and love than they will ever know.

Lastly, I would like to recognize a few key individuals at ECU Brody School of Medicine who always greeted me with genuine kindness and willingness to assist in any way possible: Dr. Richard Franklin, Dale Aycock, my favorite IT guys Robert Alves and Dave Erdmann, Jamaal Peele, Jackie Hooker, Joani Zary, Dr. Ethan Anderson and Kathleen Thayne, Dr. Weidner, Dr. Muller-Borer and Cindy Kukoly, and my committee members Dr. Mark Mannie, Dr. Qun Lu, and Dr. David Taylor. Thank you so much.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS OR ABBREVIATIONS

Aβ	Amyloid-β
Ab	Antibody
AD	Alzheimer's disease
APP	Amyloid precursor protein
BW	Blocking wash
CD	Cluster of differentiation
CSF	Cerebral spinal fluid
Cy5	Cyanine 5
DAPI	4',6-diamidino-2-phenylindole
ELISA	Enzyme-linked immunosorbent assay
FFPE	Formalin-fixed, paraffin-embedded
FITC	Fluorescein isothiocyanate
IF	Immunofluorescence
IgG	Immunoglobulin G
IHC	Immunohistochemistry
PBS	Phosphate buffered saline
PND	Postnatal day
TPBS	Phosphate buffered saline with tween

Chapter 1:

Introduction

The potential implications of developmental immunotoxicity in the etiopathology of neurodegenerative disorders, such as Alzheimer's disease (AD), have recently become an exciting area of biomedical research. Many adult diseases are now being linked to early-life disturbances of the delicate immune balance induced by environmental insults. Our primary research utilized this hypothesis as scaffolding to elucidate the role of exposure to a neuroimmunotoxicant. We evaluated the potential impact of immune dysfunction induced by exposure to a neuroimmunotoxicant during a critical period of neuroimmune development on AD pathology and disease progression in a genetically susceptible model of AD. While there may be considerable variability in environmental agents that have the ability to influence AD pathologies, we specifically chose the neurotoxic metal lead acetate due to its activating effects on microglia, the resident immune cells of the brain.

To ensure that pathologic conditions would be present, we used a triple transgenic mouse model for AD (3xTgAD). This particular model develops signs of AD neuropathologies (i.e. "plaques and tangles") as early as three to six months of age (www.jax.org). By exposing this AD model to lead during postnatal development we were able to model the genetic and environmental interface of subsets of human AD patients. We anticipate that evaluation of early-life vulnerabilities for AD will result in a better understanding of the highly complex etiologies of neurodegenerative disorders.

Developmental Immunotoxicity

Exposure to environmental toxicants early in development may lead to systematic changes to the highly regulated immune network, potentially persisting to, and causing stress in, adulthood (Leifer and Dietert, 2011). The compromised immune system is then unable to effectively maintain its

homeostatic mechanisms and inadvertently contributes to the exacerbation of adult diseases. There appears to be a fairly pervasive underlying motif in the toxicological literature that a developing immune system will have higher vulnerability to a toxicological agent than its fully matured and competent adult counterpart lacking the equivalent array of critical windows (Dietert, 2011). The increased sensitivity during these developmental critical periods can be attributed to fluctuation in immune parenchymal activity, differentiation, and extensive reliance on highly dynamic, yet strict signaling pathways. This minimal tolerance of temporal and spatial variability during these periods of vulnerability presents the opportunity for highly specific toxicological investigation into a developmental event of interest (DeWitt et al., 2012). Developmental immunotoxicity (DIT) evaluation frequently employs these critical windows, as well as the full pre- and neonatal maturational continuum, for risk assessment with high developmental specificity and accuracy (Dietert and DeWitt, 2010).

Exogenous agents considered immune-disruptive can have an assortment of impacts on the immune system, including over-excitation, suppression, and general homeostatic imbalance. Because we are focusing on "toxic" agents we often make the assumption that physiological change is adverse. It is important, also, to consider the structural, as well as functional, changes that occur, as not all deviations from a typical immune baseline present the same way (DeWitt et al., 2012).

Immune disruption has been linked to a wide assortment of disease states, varying from autoimmune/inflammatory diseases to cancer. Moreover, a new research front is emerging that implicates disrupted immune systems in neurodegenerative disease pathologies. AD, for example, is generally considered an inflammatory disease due to the severe "neuroinflammation"-induced release of proinflammatory signals, including cytokines, chemokines, proteases, and activated complement proteins by activated microglia (Lue et al., 2010). However, neuronal inflammation and neurotoxicity differ drastically from peripheral inflammation, which is why many neuropathologists adamantly refute

the all-encompassing “neuroinflammatory” nomenclature (Richartz, et al., 2005). Aguzzi et al (2013) go so far as to say neuroinflammation is not inflammatory at all, but instead degenerative, due to the restricted activation of microglia instead of the conventional inflammatory cascade. Neuroinflammation is generally regarded as deleterious, whereas classical, peripheral inflammation, under appropriate circumstances, is a major player as a first line of defense by the immune system. Peripheral inflammation acts to limit the spread of infection, remove debris, and initiate tissue repair. In one sense, neuroinflammation, due to the expression of the MHC-II surface marker on activated microglia and attraction of pro-inflammatory cytokines, is a textbook inflammatory response (Lue et al, 2010). However, to add yet another layer of complexity to the debate, microglia express two antagonistic activation states, pro- or anti-inflammatory, depending on extracellular signaling, and, unlike cells of the peripheral immune system, are not known to be divided into different subtypes. While this argument on interpretation may seem capricious and irrelevant, the most heated etiopathological dispute in AD is the involvement of microglia and the immune system in the molecular mechanisms of its pathology.

A common ground in AD research is immune dysfunction in AD, either through excessive pro-inflammatory signaling or degradation of homeostatic inhibitory signaling. Regardless of the classical definition of inflammation, AD has been correlated with the inflammatory response of the CNS time and time again (Leifer and Dietert, 2011).

Alzheimer’s Disease and Neurodegeneration

AD is a chronic, late-onset neurodegenerative disease that presents with myriad hallmark pathologies, including extracellular amyloid-β senile plaques, intracellular neurofibrillary tangles comprised of a tau protein, gross synaptic and neuritic loss, low cerebral glucose metabolism, and abnormal vasculature due to degradation of the blood-brain barrier (BBB). There is an intimate and temperamental relationship between genetic and environmental influences in disease pathogenesis.

This interplay of endogenous and exogenous effectors can also be seen in the primary dichotomy of Alzheimer's disease manifestations: familial and sporadic AD.

Although our research does not directly assess the genetic components of AD, the exploration of resultant physiological changes from genetic mutations has the potential to corroborate etiological hypotheses. AD has genetic heterogeneity, meaning mutations in numerous genes result in the same pathological phenotype (Pericak-Vance and Haines, 1995). Although AD has a high degree of mutagenic multiplicity, the autosomal dominant transmission involves susceptibilities in four ubiquitous loci: Presenilin I, Presenilin II, APOE, and APP (Pericak-Vance and Haines, 1995). The triple transgenic mouse model has mutations in the PS1 (M146V), APP (Swe), and tau (P301L) loci, which mimic a genetic predisposition by progressively accumulating senile plaques and neurofibrillary tangles. Incorporating the 3xTgAD model's overt genetic factors into our study design allows for more concise exploration of the environmental influence on AD pathological severity, essentially eliminating the confounding ambiguity of potentially unknown genetic influences. This reasoning is also justification for not including a wildtype control group in our experimental design, although future research may utilize such a comparison.

Sporadic AD is far less predictable. As the name suggests, the disease develops with no discernable pattern between individuals lacking typical genetic inheritance, and can differ in both onset and severity. Sporadic AD research is garnering a more intense research focus than its genetic counterpart due to the etiologic possibilities and numerous exploratory tangents. The experiment we conducted was designed with the understanding that non-stereotyped progression could be attributed to exogenous agents, e.g. neurotoxicants and immunotoxicants. From this point on we will refer to sporadic AD as simply Alzheimer's disease, as our particular research is aimed at the combination of environmental, rather than solely genetic, effects on the exacerbation of AD.

Etiopathology

Discordant hypotheses debating the precise mechanism by which the AD pathologies originate implicate a variety of possible culprits, including glial deposition of misfolded proteins, neuritic and somal degradation, and global oxidative and lysosomal stress. Our mechanistic focus, however, is on dysfunction of the neuroimmune parenchyma, and impacts on the protein amyloid-β. We chose not to explore the etiology of the tau protein, which accumulates and forms disruptive fibrillary tangles within neurons because the triple transgenic murine model (3xTgAD) develops amyloid-β (Aβ) pathologies prior to development of neurofibrillary tangles (Oddo et al., 2003b). Likewise, the conformational changes in tau protein are not detectable until about 12 months of age, which was outside of our experimental time frame (Oddo et al., 2003a). This is complementary to the prevailing hypothesis that implicates amyloid-β as the primary catalyst for pathogenesis, and, as we are interested in the early stages of disease, allows us to disregard tau pathologies in our focused methodology.

There is moderate evidential support for the neuroinflammation theory, which states that neuritic and synaptic deterioration seen in early stage AD has a direct impact on the localized inflammatory response (Richartz, et al., 2005). The triple transgenic mouse model used in our research has been shown to exhibit considerable synaptic and neuritic degeneration preceding any detectable senile plaque formation or tau neurofibrillary tangles (Oddo et al., 2003a). However, as previously mentioned, the term “neuroinflammation” comes bearing a variety of definitions and implications as to the beneficial versus deleterious effect of this non-classical inflammatory response.

Etiopathogenesis in AD is neither clear-cut, nor well-understood. However, recent strides regarding the very first signs and symptoms of disease have begun to explore the exact sequence of events and potential therapeutic targets.

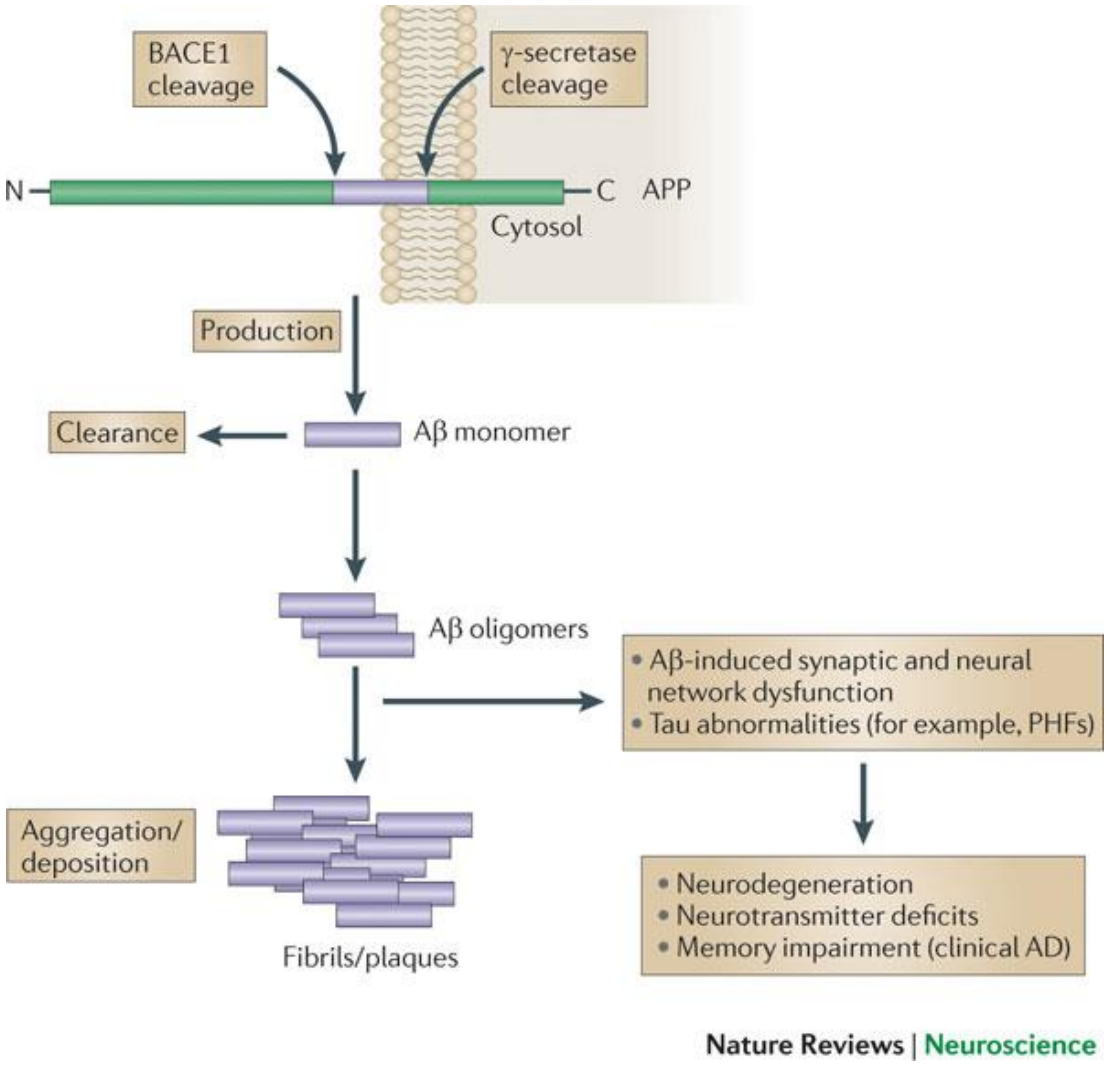
Amyloid-β

A vast majority of researchers subscribe to the Amyloid Cascade Hypothesis (ACH), a molecular model for AD etiopathology that states that the catalyst for all other pathologies is the net increase and accumulation of both soluble and insoluble amyloid-β. Recent investigations of the ACH have begun to delineate the astounding mechanistic complexity of this highly nuanced disease.

Amyloid-β is a broad term used for a protein that has the ability to fold in on itself in beta-pleated sheets, which then self-aggregate, typically in the extracellular environment (Figure 1). These polymers have appeared in a variety of pathological states, including traumatic brain injuries and prion diseases (Baglioni et al., 2006). Amyloid-β can deposit extracellularly as soluble oligomers, protofibrils, and insoluble fibrils, the latter of which contribute to senile plaque formation. As the disease progresses, the total amount of amyloid-β over-accumulation is equal to the production and deposition of the protein minus its overall clearance. The imbalance of deposition and clearance stimulates various mechanisms for amyloid-β over-accumulation. One hypothesis associated with over-production implicates changes in amyloid precursor protein (APP) secretase activity, the protease involved in APP cleavage (Mawuenyega et al., 2010). However, both familial and sporadic AD have been shown to exhibit no significant differences in the length of the dominant protein isoform (Aβ1-42 and Aβ4-42) of senile plaques, nor a skew towards isoform dominance (Portelius et al., 2010). This congruency in insoluble isoform could be due to the dysfunctional activation of the unfolded protein response (UPR) in the endoplasmic reticulum, which typically reduces the accumulation of misfolded proteins and whose upregulation is seen in AD individuals (Hoozemans et al., 2005). While plaque etiopathological theory is an intriguing and highly contested area of research, our own experimental design was created with a bias towards early-stage etiologic neuroimmune dysfunction associated with toxicant exposure. Thus, we focus our attention to the primary basis of amyloidogenesis and the associated hypotheses.

Large portions of ACH supporters assign low order soluble aβ isoforms with an integral role in amyloidogenesis. The soluble oligomeric form of aβ has been shown to be particularly neurotoxic, promoting degradation and ultimate dysfunction at both neuronal synaptic endings and dendritic spines (Lambert et al., 1998; Li et al., 2009). However, a recent study by Watt et al. (2013) has shown that the dimerization of aβ can be induced by sodium dodecyl sulphate (SDS), demonstrating that protein environment is intimately correlated with its structure, function, and subsequent neurotoxicity. While the soluble isoform may have the capability to be neurotoxic, an umbrella statement implicating low order aβ isoforms as the catalyst for AD is at the far end of data extrapolation and should be considered as thoroughly context dependent.

Figure 1. Molecular mechanism for amyloid-β production and the Amyloid Cascade Hypothesis (ACH). BACE1: β-site APP cleavage enzyme-1; APP: amyloid precursor protein; PHFs: paired helical filaments; AD: Alzheimer's disease. (Paolo and Kim, 2011).


BACE1 cleavage
γ-secretase cleavage
N
C
APP
Cytosol
Production
Clearance
Aβ monomer
Aβ oligomers
• Aβ-induced synaptic and neural network dysfunction
• Tau abnormalities (for example, PHFs)
Aggregation/ deposition
Fibrils/plaques
• Neurodegeneration
• Neurotransmitter deficits
• Memory impairment (clinical AD)
Nature Reviews | Neuroscience

Neuritic Dysfunction and Synaptic Degradation

At the risk of getting lost in theory, we need to take yet another step back to analyze the contrasting and varied hypotheses regarding the first signs of Alzheimer's pathology and molecular predisposition to disease. A fairly encompassing review by Fiala (2007) proposed dystrophic neurites, so called for the abnormal shape and indistinguishable typical neuronal structures, as the initiators of amyloidosis. This hypothesis cites the pervasive correlation of axonal damage and plaque formation in a variety of disease states (Fiala, 2007). Where and why the abnormal protein misfolding occurs is still under debate, but this review highlights an important vulnerability subject to insult in amyloid etiopathogenesis. The new question, then, is why do patients with AD experience an increase in axonal damage, and what role does the immune system play, if any, in these early stages of disease?

Continuing with a neuritic focus on AD etiopathology, a recent study by Schinder et al (2009) demonstrated a reduction in adult hippocampal neurogenesis through impaired inhibitory GABAergic signaling, which then increased overall susceptibility to neurodegenerative diseases. Adult neurogenesis is critical for learning and memory in the hippocampus, an area known for marked atrophy in late-stage Alzheimer's (Schinder et al, 2009). The hippocampus is one of the only two known areas of the brain that has the ability to support neuronal regeneration in an adult, which may be why AD presents itself in this localized region of high metabolic activity. We chose to utilize the hippocampus in our study for this particular reason, in an endeavor to expose the neuronal vulnerability for amyloidogenesis and the potential interface with the immune system.

There are numerous investigations into the effects of the peripheral and central immune system involvement in neurodegenerative disorders. We chose to focus only on innate CNS neuroimmunity and deregulation of normal homeostatic mechanisms that occur independently from the direct actions of infiltrating peripheral immune cells due to a breakdown of the blood-brain barrier. While we cannot

discount peripheral immune system involvement in the process that we were trying to model, by evaluating neuropathologies relatively early in life, we minimized the impact of peripheral macrophage activation, for example, that were unrelated to our specific research aims. Thus, we chose to manipulate the developmental environment with exposure to a model toxicant that is known to affect both the immune and nervous systems.

Neuroimmunotoxicants and the Immune System

The search for a potential experimental neurotoxicant able to produce a quantifiable physiologic change, and also capable of inducing developmental immunotoxicity, led us to lead. Our particular study had a unique set of demands for a model neuroimmunotoxicant. Not only did the exogenous agent need to be able to cross the blood-brain barrier to produce quantifiable results in the CNS, but the effects had to be pertinent to and capable of detecting changes in Alzheimer's pathologies.

From a developmental immunotoxic standpoint, lead acetate was an ideal candidate. Lead neurotoxicity has been exhaustively researched following the emergent correlation of exposure with cognitive and behavioral deficits in children. A study by Zurich et al (2002) demonstrated that increased uptake and accumulation of lead not only occurred in a developmentally-dependent manner, but also had profound effects on neuronal and glial gene expression. Furthermore, there was marked activation of microglia following lead exposure, which could act as a quantitative analytical tool for neurodegeneration, inflammation, and amyloid-β accumulation endpoints (Zurich et al, 2002).

Lead acetate exposure has been linked to upregulation of the APP gene, the innate transmembrane protein seen in healthy as well as diseased brains (Basha et al, 2005). The proteolytic cleavage of APP produces two common isoforms of the resultant amyloid-β fragments, Aβ42 and Aβ40, referred to collectively as amyloid-β; these insoluble fibrils then readily accumulate and constitute the

major component of senile plaques. The most common isoform, Aβ42, is more frequently associated with plaque formation, whereas Aβ40 presents with greater numbers in amyloid angiopathy (Mawuenyega et al., 2010).

Abnormal immune-mediated clearance of protein rather than increased production may also act as the source of Aβ over-accumulation. Disruptions in normal immune recognition, phagocytosis, and transport, both via cerebrospinal fluid (CSF) and across the blood-brain barrier, are among a few of the most frequently studied processes (Mawuenyega et al., 2010). CSF transport is an interesting hypothesis, which also ties into disruptions in circadian rhythm, seen in late stage AD patients. An increase in CSF movement during the night assists in normal homeostatic processes. However, these individuals have less diurnal and more nocturnal activity, disrupting sleep patterns and normal CSF flow (Duncan et al., 2012). Cerebrospinal fluid transport may have a role in decreased amyloid-β clearance, but a study by Duncan et al. (2012) linked this disrupted sleep-wakefulness rhythms to pathologies in the hypothalamic suprachiasmatic nucleus. Future studies may help elucidate the mechanistic link between CSF flow and early immune disruption of adult disease.

A continuation of the deposition vs clearance equation then turns our attention to other forms of inherent immune cleanup in the CNS. Neuroglia are known to provide scaffolding, nutritional support, and removal of harmful metabolites and cellular debris. But how can passive, reactionary glial cells protect the brain without help from the circulating immune system? There is one cell at the interface of the nervous system and immune system capable of initiating a full scale attack on a CNS insult: microglia.

Microglia and the Neuroimmune System

The brain and spinal cord are home to microglia, arguably the most interesting and diverse cells in the body. Microglia are the resident immune cells of the CNS; where the immune system and nervous system coalesce, there are microglia-exhibiting properties of both. Thought to originate from the mesoderm, the microglia precursors migrate during embryonic and early postnatal stages (Pocock and Kettenmann, 2007). However, the rate at which microglia colonize the developing brain differs with sex. Various studies utilizing rat models revealed twice as many microglia with a more active morphology early in postnatal development for males, and a parallel, later increase in females during adolescence (PND30-60) (Lenz et al., 2013; Schwarz et al., 2012). Because of this sexual dimorphism during a critical window of microglial development we separated males and females within treatment groups for data analysis. After migration, the resident microglia are fully confined within the blood-brain barrier, and peripheral, circulating microglia only infiltrate the CNS during highly osmotic or pathologic states (Graeber and Streit, 2010).

Until a few decades ago, the enormous microglial diversity led many scientists to believe a single cell could not encompass all observed morphogenic and functional characteristics, and only a multiple cell type model could explain the variety. Although there is a graded continuum of morphologies, we focused on the two extremes: ramified and amoeboid (Figure 2). Ramified microglia are considered dynamic sentinels, and have relatively small somas with long cytoplasmic projections, which are used to actively monitor its microenvironment. Under a light microscope these cells appear most like other neuroglia, such as astrocytes, and carry many of the same surface markers. Likewise, a ramified microglial cell will typically perform protective and homeostatic functions in the CNS. Activated, or amoeboid-like microglia, are at the opposite end of spectrum and will exhibit a large, inflamed soma with the retraction of its thin projections to aid in motility and release of signaling molecules. These activated microglia can perform a variety of functions, including initiation of the neuroinflammatory response, phagocytosis of pathogens, and the excision of superfluous synapses during brain

development. This macrophage-like morphology also mimics macrophage-like function, surface antigens, and mobility. These cells essentially transition from neuroglia to immune cells, depending on the variety of microenvironmental signals they receive.

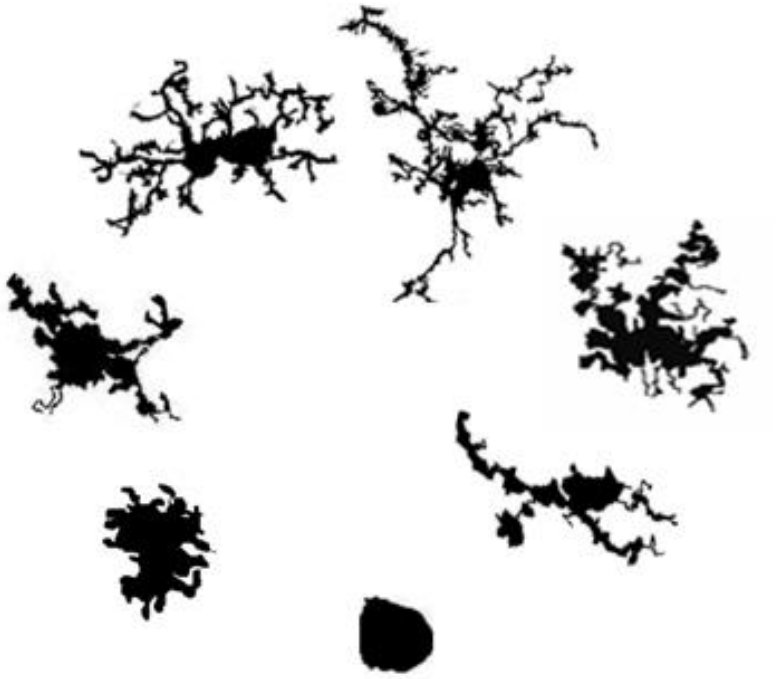
While neuronal cross-talk and molecular signals dictate much of the microglial response, there are two generally accepted forms of activation (Figure 3). The M1 state, or classical activation state, is considered proinflammatory, characterized by production of cytokines, chemokines, prostaglandins, and toxic intermediates, as well as antigen-presentation capabilities (Selenica et al., 2013). The M2 state, or alternative activation, is associated with increased production of IL-4, IL-10, and TGF-β, all neurotrophic in nature (Selenica et al., 2013). Another way to define these states of activation could be neurotoxic (M1) versus neuroprotective (M2). Both states are highly regulated by neuroenvironmental signaling, complicating any pathological association. Irregularities can occur virtually anywhere; neurotoxicity may dominate due to over-activation of the M1 state, or perhaps lack of appropriate meta-regulation of the M2 state.

A glial cell with this breadth of diversity is thought to have strict innate mechanisms that modulate activation. Similarly, a cell with the capability of adapting so readily to its environment is most likely highly sensitive to CNS aberrations, such as over-accumulation of amyloid-β or exposure to lead acetate, for example. As stated earlier, a disrupted immune system early in development may have deleterious effects that persist throughout the individual's lifetime, potentially causing hypersusceptibility to disease (Leifer and Dietert, 2011). The neuroimmune system is not exempt from this potential impact of development immunotoxicant exposure. Moreover, this early disruption could highlight and irritate the vulnerability that catalyzes AD pathogenesis. Studies of early-life infection on cognitive decline have shown changes in glial markers, and potential aberrant glial responses later in life

(Bilbo, 2010). Because of this we chose to analyze the microglial response, activation, and pathogenic role in our experimental design.

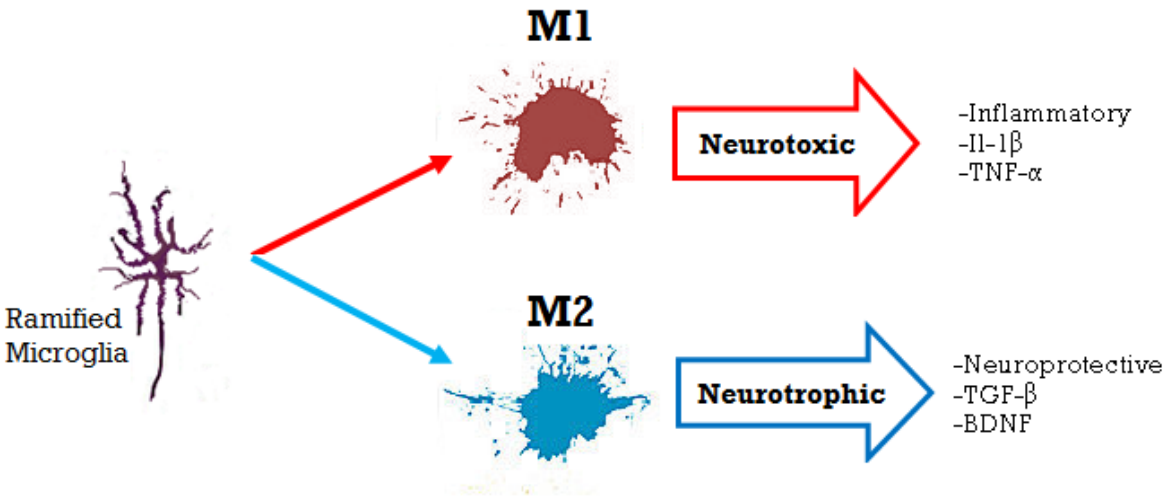
Figure 2. Morphological and phenotypic spectrum of microglial activation. (Karperien et al., 2013).

RAMIFIED



UNRAMIFIED/AMOEBOID/ACTIVATED

Figure 3. Diagram of the polarization of microglial activation states. M1, or classical activation, is considered pro-inflammatory, while M2, or alternative activation, is considered neuroprotective. Il-1β: interleukin 1-β; TNF-α: tumor necrosis factor-α; TGF-β: tumor growth factor-β; BDNF: bone-derived neurotrophic factor.


M1
Neurotoxic
-Inflammatory
-Il-1β
-TNF-α
Ramified
Microglia
M2
Neurotrophic
-Neuroprotective
-TGF-β
-BDNF

Interactions with Amyloid-β

The relationship between microglia and amyloid-β is far more entangled than a simple "phagocytizer" and "phagocytizee." There is substantial evidence showing microglial activation and association with oligomeric aβ, fibrillar aβ, and all senile plaque phenotypes (Cho et al., 2013; Koenigscknecht and Landreth, 2004; Combs et al., 2001; Stalder et al., 1999; Paresce et al., 1996). The implication is that amyloid-β induces an activated microglial phenotype, directly or indirectly. Microglia possess the ability to phagocytize aβ, but it is unclear if there is greater incidence of clearance, deposition, or lack of regulation in individuals with AD (Mawuenyega et al., 2010; Bolmont et al., 2008; Hickman et al., 2008; Cras et al., 1990). We designed an experiment in which we disrupted the neuroimmune system and monitored subsequent changes to determine its role. Our primary goal was to elucidate the role of a developmentally disrupted neuroimmune system in the exacerbation of AD pathologies. With this in mind, we anticipate that the exposure to lead during a critical window of development for microglia would result in an altered immune system persisting in to adulthood, and a subsequent increase in the pathological severity of AD, as assessed by amyloid-β load and incidence, in a genetically prone murine model.

Chapter 2:

Materials and Methods

Animal Handling and Dosing

All animal handling, dosing, and euthanasia was carried out in accordance with procedures approved by the East Carolina University Brody School of Medicine Institutional Animal Care and Use Committee (IACUC). Triple transgenic mice (3xTgAD) homozygous for all three mutations (B6;129-*Psen1*^{tm1Mpm}Tg(APPSwe,tauP301L)1Lfa/Mmjax) were used due to the spontaneous generation of Alzheimer's-like plaque and tangle pathologies (Charles River, Raleigh, NC). Transgenic mice were then paired for mating by the ECU Department of Comparative Medicine animal care staff and, once confirmed pregnant, dams were transferred to the Brody Animal Care Facility. On the day of birth, considered postnatal day one (PND1), pups were sexed and culled to six animals per dam, balanced by sex if possible. All mice were maintained on a 12:12 hour light/dark cycle and fed and watered *ad libitum*. If considered healthy following the 10 day dosing regimen, the neonates were weaned at PND21, placed into same sex cages of no more than three animals per cage with the appropriate enrichment, and monitored for signs of barbering and hydrocephaly.

Offspring were gavaged from PND5-15, a critical window of development for microglia in a rodent model. Experimental dosing procedures were modified for neonate oral gavaging using a bent stainless steel 1.25mm 24G feeding needle with ball tip (ref# 7900, Braintree Scientific, Inc., Braintree, MA) and placed in the pup's mouth to stimulate a suckling response rather than a potentially harmful full intubation. Not only does this technique ensure consistency in dosing concentrations, but it is also a common practice in the laboratory that has been shown to provide a successful route of exposure in preweaning toxicity testing (Zoetis and Walls, 2005; Moser et al., 2005; Butchbach et al., 2007).

The dosing solution consisted of either sterile water for control or lead (II) acetate (lot# MKBN8098V, Sigma-Aldrich, St. Louis, MO) dissolved in sterile water for treated animals. The dosing concentration was set at a physiologically-relevant 100ppm, with 0.1mL dosing solution per 10g daily body weight, a concentration recommended in rodent model toxicity and risk assessment (Zoetis and Walls, 2005; Moser et al., 2005). New batches of dosing solution were made each week. Dams were euthanized at weaning (PND21), and pups were weighed and monitored daily until the predetermined terminal endpoint. A total of 24 dams and 97 offspring were euthanized and assessed for pathological endpoints.

Tissue Collection and Preparation

Three terminal endpoints were chosen, PND 50, 90, and 180. These endpoints were chosen based on the anticipated pathological detection of plaques and tangles for the 3xTgAD mouse model at approximately six months. One neonate of each sex per litter, if available, was euthanized at the pre-determined terminal endpoint. Following decapitation a sagittal incision was made down the cranium, and the skull and meninges were carefully peeled back and away from the brain. The full brain was carefully removed to avoid excess peripheral blood contamination. We placed the tissue into a petri dish with phosphate buffered saline (PBS) and, using a coronal scalpel cut, removed the cerebellum and olfactory bulbs for consistency. The two hippocampi were extracted using a novel technique to isolate the hippocampal regions CA1, CA3, and dentate gyrus (Hagihara et al., 2009). Regardless of its hemispheric origin, one hippocampus was snap frozen at -80^{0}C for ELISA analysis. The second hippocampus was placed directly in a scintillation vial filled with 10% neutral-buffered formalin for 24-hour fixation, then placed in a tissue cassette submerged in 70% ethyl alcohol until subsequent embedding in paraffin wax.

The formalin-fixed paraffin-embedded (FFPE) hippocampi were sliced on a rotary microtome at 5μm for offspring and 10μm for dams, floated in warm water (~40^0C) and ethyl alcohol (50%) baths, and mounted on Unifrost Plus slides with Azerbond (cat #EMS600W+, Azer Scientific, Morgantown, PA), four sections per slide and four slides per animal. Slides were left to dry overnight on a covered rack and stored in a slide box until IHC and IF staining. Remaining brain tissue was immediately placed in buffer after hippocampal extraction to be homogenized for flow cytometric analysis.

Enzyme-linked Imunosorbent Assay (ELISA)

To detect varying levels of the protein amyloid-β within the hippocampus, an enzyme-linked immunosorbent assay (ELISA) was performed on the snap frozen hippocampi. The hippocampus was thawed, homogenized with phosphate buffered saline and tween (TPBS) in a 5mL Tenbroeck glass homogenizer, and weighed. A commercially available BetaMark™ Beta Amyloid ELISA kit (cat #SIG-38954-kit, Covance, Dedham, MA) as per manufacturer's instructions, was used to assess protein concentrations. Standard curve assessment and data analysis was carried out based on the absorbance readings obtained from the hippocampus samples and standards included in the ELISA kit.

Flow Cytometry

Brain tissue sans hippocampi was homogenized in a 15mL Tenbroeck glass homogenizer containing FACS buffer (3% FCS + 0.1% sodium azide + 10 mM EDTA in PBS), passed through a 70μm strainer, and centrifuged at 3000 RPM. To remove myelin, the pellet was resuspended in 30% Percoll (#17-0891-02, GE Healthcare, Uppsala, Sweden), which has been shown to produce a higher yield of live cells in comparison to other demyelinating methods, e.g. sucrose and anti-myelin beads (Nikodemova and Watters, 2012).

After centrifugation at 3000 RPM, the supernatant containing the buoyant myelin and excess tissue debris was carefully removed via manual bulb suctioning with a 1mL glass pipette. The subsequent pellet containing microglia was resuspended in buffer and passed through a 40μm filter to produce a single-cell suspension and increase purity. A third spin in the centrifuge (3000 RPM) and removal of the supernatant allowed for resuspension in the blocking buffer (FACS buffer + 5% normal mouse serum +5% normal rat serum + 1% Fcr block [polycloncal anti-mouse IgG CD16/CD32; stock #14-0161, eBioscience, San Diego, CA]).

Following another centrifugation/resuspension wash, the suspension was incubated with two monoclonal antibodies, CD11b-APC (1:100 dilution; ab25482, Abcam, Cambridge, MA) and CD45RB-FITC (1:100 dilution; cat# 11-0455-82, eBioscience Inc., San Diego, CA). These surface antigens were chosen for their microglial specificity and consistent use throughout the literature (Sedgwick et al., 1991; Ford et al., 1995; Stevens et al., 2001; Parney et al., 2009; Nikodemova and Watters, 2012). The antigen CD11 is a common surface marker for monocytes/macrophages, as well as microglia, which is why a second antigen was employed for immune specificity. Microglia can take on a macrophage-like phenotype under certain conditions and function in a similar manner, which is why both have the surface antigen CD11. However, CD45 is a leukocyte-common antigen and identifies cells with a myeloid lineage. The degree to which microglia express these antigens has been associated with phenotypic changes that occur upon microglial activation (Stevens et al., 2001).

After the dual-antibody incubation (one hour at 4^0C), another centrifugation/resuspension wash cycle was followed by propidium iodide (1 μg/mL) live cell viability staining, and immediate analysis using a BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ). A total of 10,000 events were collected per sample and the emission wavelengths for each fluorophore were detected using the FL1 (FITC) and FL4 (APC) lasers. Gating of the representative dot plots consisted of a consistent quadrant

marking scheme. Analysis of microglial activation was performed through comparison of the ratio of the upper right quadrant (high expression of CD45/CD11b, activated microglia) to the lower left quadrant (low expression of CD45/CD11b, resting microglia).

The flow cytometry dependent variable, "ratio," was the value of the upper right (UR) quadrant of the flow output divided by the lower left (LL) quadrant value, meant to represent the number of activated (amoeboid) microglia (UR) corrected for ramified microglia and other macrophages (LL).

Immunohistochemistry

Morphological changes associated with microglia activation were qualitatively assessed using immunostaining against the ionized calcium-binding adaptor molecule-1 (Iba1) surface antigens found on microglia. The FFPE hippocampi tissue mounted on Superfrost Plus slides were dewaxed and rehydrated through sequential washing with Histo-Clear II (cat# 64111-04; Electron Microscopy Sciences, Halfield, PA), ethyl alcohol (100%, 95%), and PBS. Antigen unmasking was accomplished using a heat-mediated citrate buffer system to break the protein cross-links generated during tissue fixation, improving antigen binding and specificity. The slides were then submerged in 0.3% hydrogen peroxide for 30 minutes to quench endogenous peroxidases. This reduces background staining by "capping" peroxidase inherently found on the cell surface so it cannot bind with the diaminobenzidine (DAB).

All immunostaining of the FFPE hippocampal slides was carried out using the Sequenza-Coverplate system (Thermo Scientific, Waldorf, Germany), which combines surface tension, capillary attraction, and positive displacement to hold minimal amounts of reagent directly against the tissue without drying. Slides were carefully pressed against Coverplates saturated in TPBS to eliminate air bubbles, and inserted into individual Sequenza slots. All subsequent reagents were directly dispensed into the top of each slide well, ensuring consistency in staining quality with correct assembly. Blocking

with diluted normal serum was followed by a 60 minute/overnight incubation with the primary antibody, rabbit polyclonal anti-Iba1 (1:500 dilution; stock #019-19741, Wako Chemicals USA, Inc., Richmond, VA).

After incubation with the primary antibody, slides were washed with TPBS, and the biotinylated secondary antibody (diluted and prepared according to ABC Vectastain kit protocol) was applied for 30 minutes. The final labeling step was a biotinylated secondary antibody with an avidin peroxidase capable of oxidizing DAB for staining visualization. Slides were then stained using a DAB kit (Vector Laboratories Inc., Burlington, ON, Canada) and counterstained with Harris' Alum Hematoxylin (lot# 9290, Crescent Chemical Co., Inc., Islandia, NY). Following removal from the Sequenza system, the slides underwent a final wash sequence of PBS, ethyl alcohol (95%, 100%), and Histoclear. All slides were coverslipped with Permount (lot#107422, Fisher Scientific, Fair Lawn, NJ) and left to cure overnight prior to light microscopy visualization and manual morphometric analysis.

The immunohistochemistry dependent variable, "IHCV," was calculated by taking the number of amoeboid microglia per counting frame divided by the total microglia per frame, multiplied by the number of amoeboid microglia per frame, and multiplied again by the average total number of microglia for that particular sex, dose, and age group. This arbitrary value was designed to give greater importance to amoeboid microglia, which are easier to detect via IHC than ramified microglia, and likely a more accurate representation of microglial activation.

Immunofluorescence

Simultaneous imaging of both amyloid-β and microglia was accomplished through high-resolution immunofluorescent staining and laser scanning confocal microscopy. This method of visualization utilizes point-to-point laser scanning of the specimen, called optical sectioning, to create a

three dimensional image with incredibly high resolution and clarity to profile the overall surface, as well as interior structures.

The FFPE slides were dewaxed and rehydrated using the same HistoClear, ethyl alcohol (100%, 95%), and PBS sequential wash as the IHC protocol. Similarly, antigen unmasking occurred via heat-mediated citrate buffer. All subsequent immunofluorescent staining was carried out using the Sequenza plate system. Sequenza coverplates were immersed in TPBS rather than PBS to promote membrane permeabilization and stripping of superfluous antibodies for optimal staining.

The complexities of staining with two primary and two secondary antibodies raised in separate hosts required strict sequential blocking wash steps (Table 1). This system was created to ensure highest binding and specificity, while reducing self-adhesion to blocking proteins. The first block (BW1) consisted of TPBS and donkey serum (10%), the host of the first secondary antibody. After a 30 minute incubation at room temperature the first primary antibody, anti-Iba1 (1:1000 dilution; goat pAb to Iba1, ab107159, Abcam, Cambridge, MA), was applied for one hour, or overnight at 4^{o}C if needed. All following incubations occurred at room temperature removed from light due to the sensitivity of the secondary antibodies. After a wash with TPBS, the slides were incubated for one hour with the first secondary antibody, anti-goat IgG-FITC (1:1000 dilution; donkey anti-goat FITC-conjugated polyclonal IgG, ab6881, Abcam, Cambridge, MA).

The second blocking wash (BW2), which consisted of TPBS, donkey serum (5%), and goat serum (30%), was applied for 30 minutes at room temperature, protected from light. The following one hour incubation was with the second primary antibody, anti-beta amyloid (1:1000 dilution; rabbit pAb to beta Amyloid, ab2539, Abcam, Cambridge, MA). After a wash with TPBS, there was a 30 minute incubation with the final secondary antibody, anti-rabbit IgG-Cy5 (1:1000 dilution; goat anti-rabbit Cy5-conjugated polyclonal IgG, ab97077, Abcam, Cambridge, MA). Cell nuclei were stained using DAPI (300 nM dilution;

D3571, Life Technologies, Eugene, OR) for 2-5 minutes and washed three times with TPBS. Following removal from the Sequenza racks the slides were left to dry completely before coverslipping with ProLong Gold antifade reagent (P36930, Life Technologies, Eugene, OR) or with a homemade anti-fade aqueous mounting media (glycerol, phosphate buffer solution-pH 7, and *p*-phenylenediamine hydrochloride). For long term storage, edges of the slides were sealed with Permount if mounted with the homemade anti-fade media and stored upright in a slide box after curing.

Confocal viewing was performed with a Zeiss LSM 700 confocal microscope in the Brody Core Imaging Center. The following emission wavelengths were used for tri-color visualization: DAPI – 405nm, FITC – 488nm, and Cy5 – 639nm. All images were captured at 10x magnification, with the plaque counting frame of 640.17μm by 640.17μm. Plaques were characterized based on number and relative morphology following a subjective scale guideline, shown in Figure 4.

The immunofluorescence dependent variable, "ψ," was calculated by multiplying the qualitative plaque scale value (1-5) by the intensity ratio (amyloid intensity bit value divided by 256 total bits) and again by the square root of the plaque area. This arbitrary value was designed to assess the maturity of the plaque, taking into account the amyloid load and giving less importance to the plaque area, a less reliable aspect of plaque maturity based on the region of hippocampus analyzed and tissue thickness.

Statistical Analysis

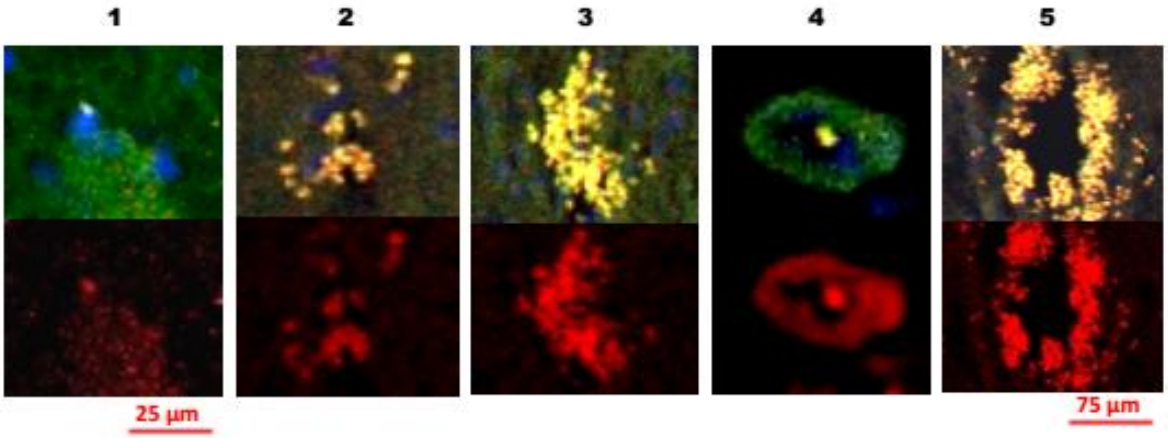
Analysis of all data was accomplished with the Statistical Analysis System (SAS Institute, Cary, NC). Flow cytometry, immunohistochemistry, and immunofluorescence data were analyzed using a three-way analysis of variance (ANOVA) for differences in age, sex, and treatment (Flow, IF n=94; IHC n=337). When appropriate, these were then followed by individual comparisons of differences between treated and control groups, as well as differences between sexes, using t-tests. Post hoc analysis of

statistically significant differences in age, as determined by ANOVA, was accomplished using Tukey's studentized range for equal variance and least squares means t-test for age-related differences. ELISA data were analyzed for differences in age and treatment groups using t-tests assuming equal variance. $P < 0.05$ was considered significant.

Table 1. Immunofluorescence Staining Procedure. All dilutions 1:1000; Ab: antibody; TPBS: phosphate buffered saline with tween; IgG: immunoglobulin G.

Sequence	Block Wash	Primary Ab	Host	Secondary Ab	Host
Step 1	TPBS + donkey (10%)	-	-	-	-
Step 2	TPBS	Anti-Iba1	Goat	-	-
Step 3	TPBS	-	-	Anti-goat IgG	Donkey
Step 4	TPBS + donkey (5%) + goat (30%)	-	-	-	-
Step 5	TPBS	Anti-amyloid-β	Rabbit	-	-
Step 6	TPBS	-	-	Anti-rabbit IgG	Goat
Step 7	TPBS	[DAPI]	-	-	-

Figure 4. Confocal images depicting qualitative senile plaque scale. Immunofluorescent images of the gradiation of senile plaques from least mature and diffuse (1) to most mature and cored (5). Top row includes three fluorescent stains for microglia (green, FITC), amyloid-β (red, Cy5), and cell nuclei (blue, DAPI), with colocalization in yellow. Bottom row depicts the same image using only the 639 nm laser filter to illustrate amyloid load and variability with plaque maturation. Scale bar begins at 25 μm and gradually goes up to 75 μm.


1
2
3
4
5
25 µm
75 µm

Chapter 3:

Results

Flow Cytometry

There was no significant difference in microglia activation as detected by signaling of surface markers CD11b and CD45 among treatment, sex, and age through a three-way comparison of the UR:LL ratio means (Figure 5). However, post hoc analysis showed lower microglial activation in treated females than control females at PND 180. General trends towards lower activation with age were observed for both sex and treatment.

Immunohistochemistry

Phenotypic analysis of microglia activation via IHC showed significantly higher activation in treated animals at PND 50 (Figure 6). There was also higher activation in control females than treated females at PND 90. Post hoc analysis found higher activation in treated males than treated females at PND 50 and PND 90, and higher activation in control males than control females at PND 180. Overall, the age of the animal was important to microglial activation phenotypically, in regard to the exposure to lead; the older the animal the greater the phenotypic difference with both sex and treatment.

Immunofluorescence

There were no significant differences in the pathological maturity of the plaques as assessed by IF (Figure 7). More plaques were observed in treated than control males at PND 50 (Figure 8). General trends revealed a slightly higher number of plaques in treated than control animals, and in treated males than treated females. A typical, untreated 3xTgAD mouse developed plaques gradually, though

not necessarily by PND 50. Treatment with lead, however, appeared to elicit more consistent incidence of plaque pathologies at all ages, even at the earliest terminal endpoint.

ELISA

There was no significant difference in amyloid concentration, as detected by ELISA, among treatment, age, and sex (Figure 9). General trends showed higher concentrations at the earlier time points (PND 50 and 90), and a drop at PND 180.

Figure 5. 3xTgAD mouse brain microglial activation with age, sex, and treatment. Activation was assessed by the ratio of activated microglial surface markers CD11b and CD45 to ramified microglia (UR:LL) through flow cytometry. Error bars as standard error. Significant differences in treatment annotated by #. PND: postnatal day.

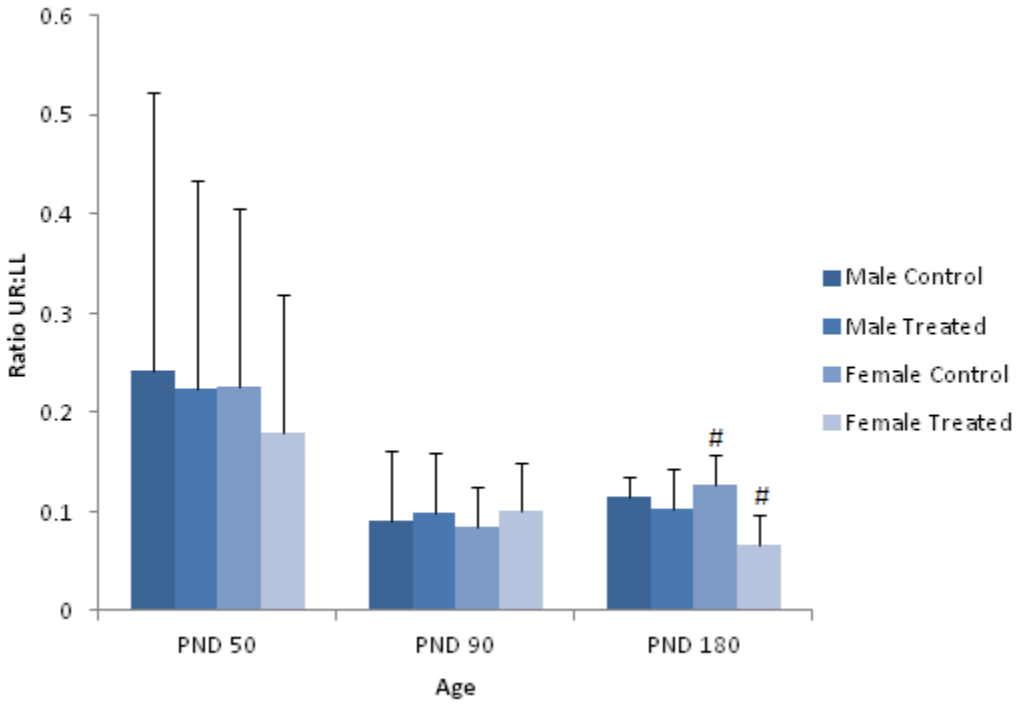

0.6
0.5
0.4
0.3
0.2
0.1
0
Ratio UR:LL
PND 50
PND 90
PND 180
Age
#
#
Male Control
Male Treated
Female Control
Female Treated

Figure 6. 3xTgAD mouse hippocampus microglial activation with age, sex, and treatment. Activation is assessed by the value "IHCV", representing the number of amoeboid microglia with respect to overall number through morphometric analysis of phenotype via immunohistochemistry (microglia immnolabeled with anti-Iba1 antibodies). Error bars as standard error. Significant differences in treatment annotated by #. Significant differences in sex annotated by *. PND: postnatal day.

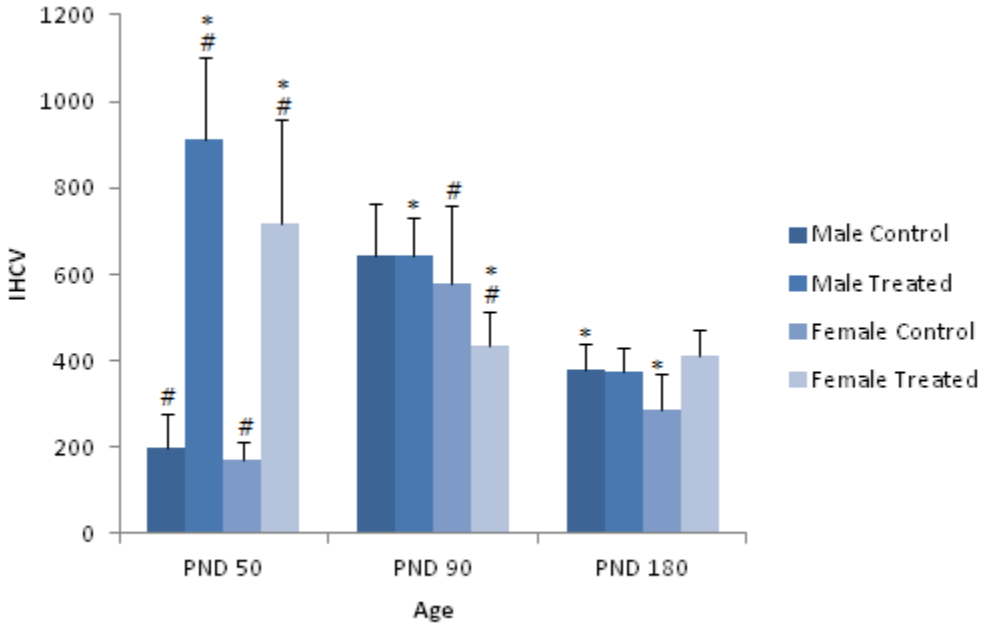

1200
1000
800
600
400
200
0
IHCV
PND 50
PND 90
PND 180
Age
Male Control
Male Treated
Female Control
Female Treated

Figure 7. 3xTgAD mouse hippocampus senile plaque maturity with age, sex, and treatment. Activation is assessed by the value "ψ", representing the overall maturity of the plaque, with respect to amyloid load, plaque size, and qualitative maturation analysis, via immunofluorescent labeling of microglia (anti-Iba1-FITC), amyloid-β (anti-amyloidβ–Cy5), and cell nuclei (DAPI). Error bars as standard error. PND: postnatal day.

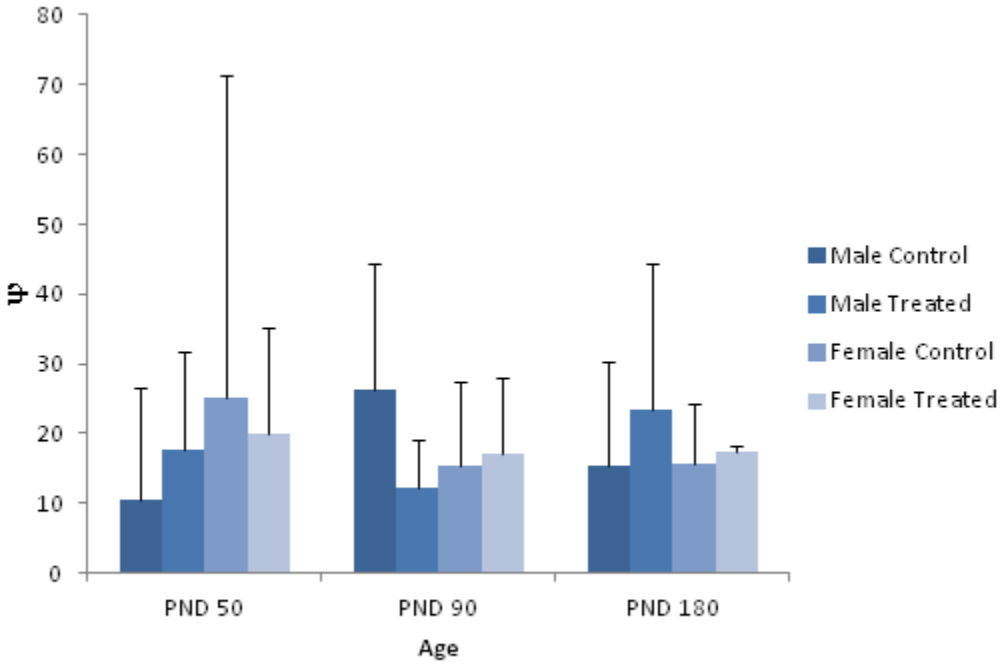

80
70
60
50
40
30
20
10
0
Male Control
Male Treated
Female Control
Female Treated
PND 50
PND 90
PND 180
Age

Figure 8. 3xTgAD mouse hippocampus number of senile plaques with age, sex, and treatment. Plaques were visualized using immunofluorescent labeling of microglia (anti-Iba1-FITC), amyloid-β (anti-amyloidβ–Cy5), and cell nuclei (DAPI). Error bars as standard error. Significant differences in treatment annotated by #. PND: postnatal day.

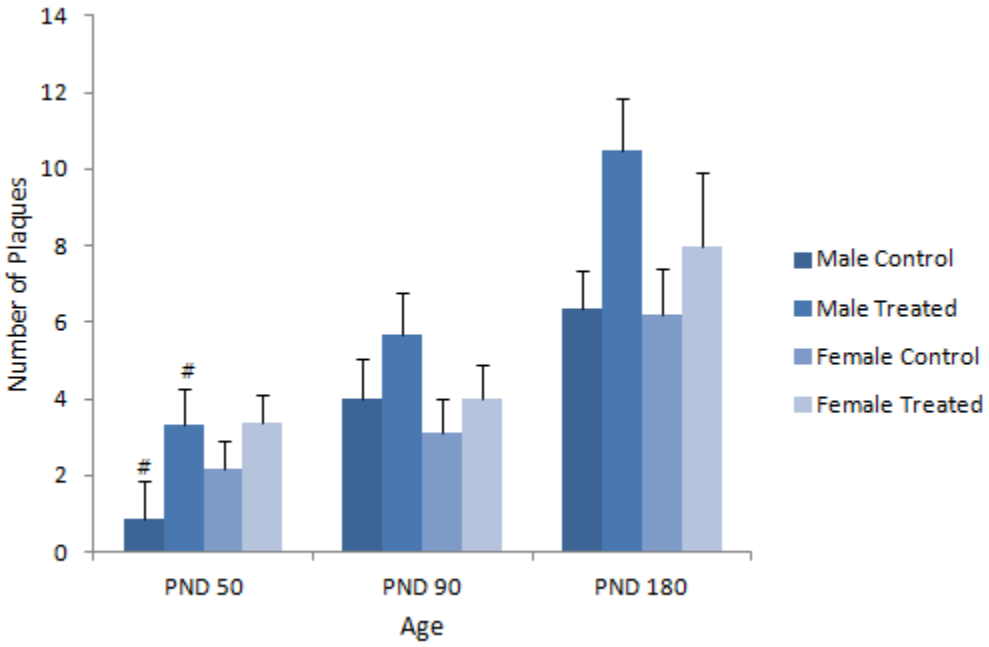
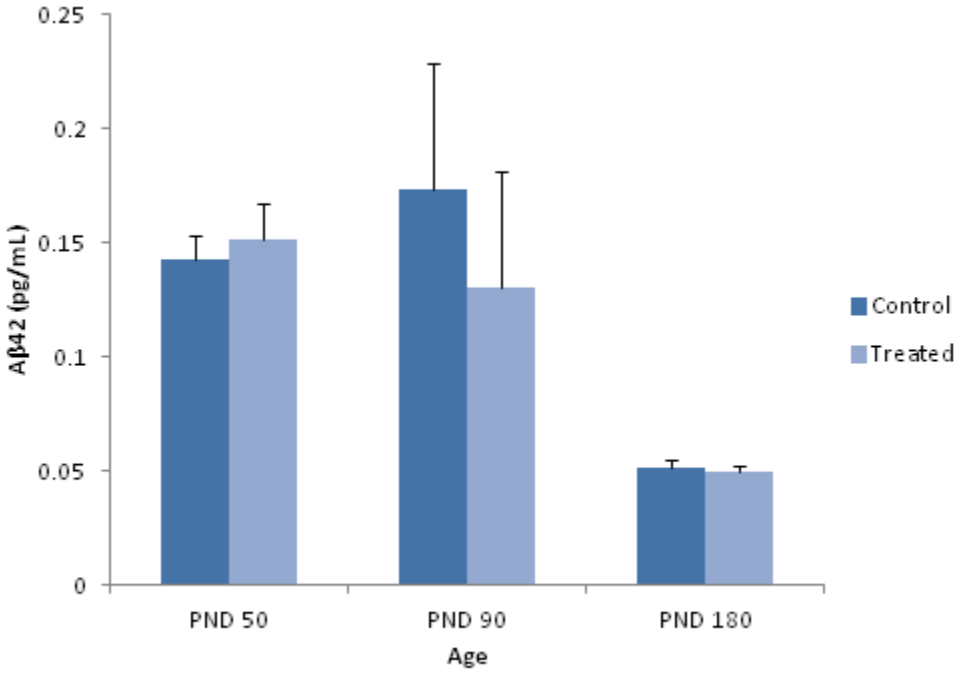

14
12
10
8
6
4
2
0
Number of Plaques
#
#
PND 50
PND 90
PND 180
Age
Male Control
Male Treated
Female Control
Female Treated

Figure 9. 3xTgAD mouse hippocampus amyloid load with age, sex, and treatment. Amyloid load was assessed through aβ42 concentration using an enzyme-linked immunosorbent assay. Error bars as standard error. PND: postnatal day.


0.25
0.2
0.15
0.1
0.05
0
Aβ42 (pg/mL)
PND 50
PND 90
PND 180
Age
Control
Treated

Chapter 4:

Discussion

The primary goal of this study was to evaluate changes in Alzheimer's pathology following developmental perturbation of the neuroimmune interface. This approach allowed us to illuminate a potentially mechanistic role of the developing immune system in the etiology of a late-onset adult disease through an extensive array of immunological analyses within the confines of a defined set of experimental parameters. Our study incorporated multifaceted approaches (i.e., flow cytometry, ELISA, immunohistochemistry, and immunofluorescence) to understand the interaction of microglia with AD pathologies and how this interaction could be disturbed by exposure to a developmental toxicant.

This experimental paradigm did not provide consistent results from assay to assay, nor was there a perfect linear correlation in lead-exposed mice between age and the severity of plaques. We can, however, still draw interesting conclusions and suggest paths for future research. The most striking significant difference was between treated and control groups in microglial morphology. A greater number of amoeboid microglia were observed in the lead exposed group at PND 50, relative to unexposed groups. However, in unexposed females evaluated at PND 90, more amoeboid microglia were observed. These results suggest that exposure to lead acetate may be transient, i.e. it produces short-term changes that do not persist, or produces various downstream cascades that are detectable at later ages. These differences can be explored, in part, by looking at the interrelation with the remaining independent variables, sex and age.

Both immunohistochemistry and flow cytometry were used to assess various aspects of microglial activation, which we then used as a gauge of an overall neuroimmune response. We chose to exclude astrocytes, the only other glial cell type associated with senile plaques, as indicators of reactive

gliosis (a parallel CNS response to toxicants). This exclusion was based on a study that demonstrated microglial activation both preceding and regulating subsequent astrocyte response to lead exposure (Zurich et al., 2002). Thus, any alteration in microglia form, function, or activated phenotypic response would be indicative of early changes in the neuroimmune system. Additional studies involving the same 3xTgAD mouse model have shown no significant microglial activation (assessed via IHC) between 60 and 180 day-old hippocampal sections (Janelsins et al., 2005). This further validates the sensitivity of microglia as an indicator of neuroimmune response to a neurotoxicant, rather than the genetic disease state, whose amyloid pathologies have shown to be potent microglial activators (Combs et al., 2001).

We chose to employ ELISA and immunofluorescence to measure changes in amyloid-β concentration and plaque involvement. We then used these fluctuations in amyloid load to parallel overall AD pathological severity based on the catalytic theory of amyloid etiopathogenesis. This is demonstrated by both the amyloid cascade hypothesis (Chapter 1), and the robust correlation of dementia in the elderly with traditional markers of protein accumulation, senile plaques, and related cytoskeletal changes (Dickson et al., 1995).

Treatment-related Differences

The early "vulnerability" in Alzheimer's disease pathogenesis we were attempting to highlight with our developmental exposure paradigm was temporally limited by critical windows of development for microglia. We hypothesized the pathogenic weak spot was during this window of postnatal exposure (PND 5-15) for a variety of reasons. First, microglia precursors are known to colonize the brain during embryonic and early postnatal stages, meaning these developing immune cells possess hypersusceptibility to environmental signals during this time frame, and were therefore more likely to be influenced by toxicant exposure (Pocock and Kettenmann, 2007). Likewise, neurotoxicity of lead is maturationally-dependent, producing more intense glial reactions and aberrations in the neuronal

microenvironments in younger individuals (Zurich et al., 2002). The focus on postnatal as opposed to prenatal exposure was twofold: i) there appears to be a skew in the literature for analyzing prenatal exposure over postnatal, giving this study an innovative edge and, ii) a study by Koo et al. (2003) demonstrated that, while prenatal environment is pertinent to overall brain health, stress during this time can be corrected by postnatal conditions, implying that the postnatal environment has greater importance and ubiquity for neurodevelopment. If there was a proverbial chink in the neuroimmune armor that could persist into adulthood, it would be during this critical window.

Sex- and Age-related Differences

The incidence of Alzheimer's disease is noticeably higher in women, who present with increased rate of cognitive decline and severity of dementia (Schmidt et al., 2008). Heterochronic discord between the sexes also has been observed, with females developing pathologies faster and earlier than males, potentially due to age-related stress to the superior frontal gyrus transcriptome (Yuan et al., 2012). This skew is also thought to exist due to estrogen depletion later in life, rather than an early developmental or genetic vulnerability, which we investigated in this study. Sexual discrepancy in the CNS is not a new concept. In fact, sexual discrepancies can be seen in all aspects of our study, from the nervous and immune systems to overall development. The oscillations in steroid hormones and gene expression may provide a critical developmental link in any pathological predilection towards a particular sex.

Male and female brains differ macroscopically, as well as microscopically (e.g. behavior and gene expression, respectively). The differentiation extends to developmental timing as well. Investigations into related epigenetics indicate that these inherent differences are organized perinatally, and permanently influence adult behavior through myriad downstream cascades (Lenz et al., 2012). These post-genetic effects are conducted by the neuroimmune system. Again, microglia demonstrate

their diversity by acting as vital components to the normal development and sexual differentiation of the brain. Increased prevalence of activated phenotypes during development can be linked to the restructuring of neuronal circuits, axonal migration, and high levels of metabolism and metabolic signaling. However, the number and activation of male microglia is higher during early postnatal development (PND 4), whereas female microglial activity crests later in development (PND 30-60) (Schwarz et al., 2012). This suggests that male microglia may be more sensitive to exogenous influences than female microglia during our experimental exposure window. Our data showed the gradual decrease in microglia activation from PND 50, when female activation is anticipated to be exceptionally high, to PND 180, when both sexes should, and do, show more relaxed and ramified phenotypes. The trend over time was seen in the gradual decline in expression of activated surface markers (Fig. 5).

Immunohistochemistry illustrated a similar overall temporal decrease in immune response (Fig. 6), but with a considerably weaker response in females at all terminal endpoints. A wildtype control may have helped us to understand this phenomenon, but we hypothesize that the female response at PND 50 is accurate and normally elevated, and rather the difference is due to hyperstimulation of the male response due to the early immune disruption. This idea is supported by the significant differences in sex-related activation only in animals exposed to lead (Fig. 6). Likewise, there were sex differences between PND 50-90 and PND 50-180 activation surface markers in untreated brains and PND 50-90 for treated brains, but no differences in sex at the PND 180 time point. Males that experienced an unperturbed activation crest and normal immune development exhibited the same gradual decline as control females. We can expand our hypothesis to include a sexually dimorphic caveat: increased male microglial activation resulted from lead exposure, with a subsequent persistent immune response (i.e., activated microglia), and contributed to an increase in AD pathologies in male brains. This counteracts the typical female skew in pathology, and reduces detectable sex differences in AD severity in our model. This caveat is supported by the lack of significant differences in control male and female amyloid

load, as assessed by immunofluorescence (Fig. 7). Thus, we can deduce that lead exposure had a greater activating effect on male microglia during this critical window of development.

Immune Involvement in Early Stages of Disease

There were no significant differences in amyloid load as detected by ELISA and IF, but confocal imaging of cell nuclei, microglia, and amyloid-β reaffirmed the immediate involvement of the neuroimmune system at various stages of pathological progression. The role of microglia in amyloid accumulation in AD pathology is not well-understood. However, we were able to demonstrate the correlation of microglia with senile plaque formation through three dimensional immunofluorescent imaging (Fig. 10). Some researchers argue this association with senile plaques is comparable to a massive clustering of dead cells and protein, as supported by the abnormal cell nuclei in the associated dystrophic neurites, rather than a counterattack from the active immune system (Streit 2010). Additionally, it has been proposed that axonal damage, synaptic dysfunction, and age-related neuronal death are the primary catalyst to amyloidosis, with microglia acting as a tardy and feeble attempt to clear the debris via phagocytosis, and are rapidly overwhelmed and dystrophic themselves (Streit et al., 2009).

We propose a parallel hypothesis. AD is a disease of the elderly, with much of the pathologies culminating from a perfect storm of normal age-related deficits and, occassionally, genetic influences. While searching for a fetal basis of adult disease and in attempt to elucidate a developmental immune vulnerability, we instead explored various potential targets, whose individual effects were not statistically significant. If we subscribe to the theory that amyloid is not the sole catalyst for other pathologies then microglia, as processors of amyloid-β, become secondary and imply no immune component in etiopathogenesis. However, microglia are the only immune cells intrinsic to the CNS, with

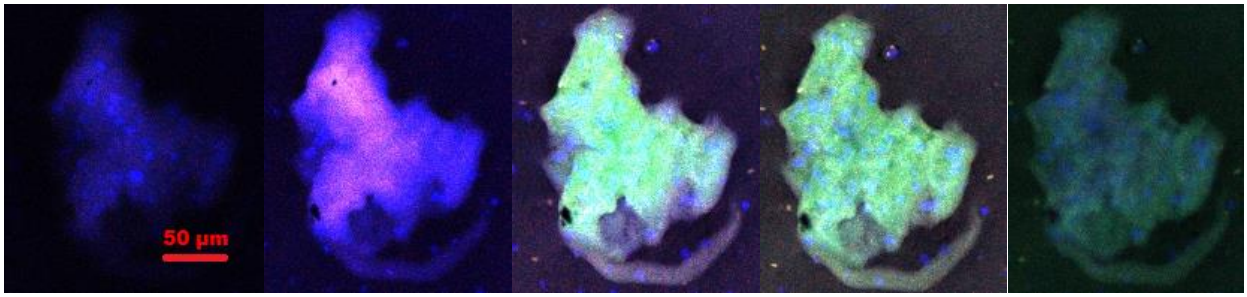
such an extensive array of functions, that there is a high probability that they are involved in the early exacerbation of neuronal dysfunction seen in AD individuals.

Preceding selective neuronal loss, however, is the alteration of neurogenesis in the hippocampus (Lazarov and Marr, 2013). The ACH returns yet again in the various studies that implicate APP fragments as having an amyloidogenic pathway and significant decreases in adult neuogenesis in murine models for AD, including our own (Mu and Gage 2011; Rodriguez et al., 2008; Donovan et al., 2006). Furthermore, this disturbed adult neurodegeneration has been propsed as the single neuronal vulnerability for early impaired cognition and decreased capacity for neuronal rebound (Schinder and Morganstern, 2009). The cell type responsible for directing the development of neuronal circuits and proliferation of hippocampal progenitor cells is, of course, microglia. So we have come full circle to re-implicate the neuroimmune system in the early vulnerabilities for Alzheimer's disease.

We hypothesize that the early neuronal degeneration preceeding plaque and tangle pathologies is correlated to disturbances in the neuroimmune system, be it from abnormal development or age-related microenvironmental stressors, such as calcium imbalances that occur in a typical aging paradigm. Microglia have the capacity to undergo either neuroprotective or neurotrophic activation, which could occur in sequence or simultaneously, giving rise to the overt complexity of their role in AD. These activated microglia may then respond to distress signals from the dying neurons inappropriately, or transition to an inappropriate response due to improper signaling cues. In a typical AD model, the microglia may become dystrophic themselves and fail to perform homeostatic functions, perturbing the delicate balance in the extracellular environment and promoting a positive apoptotic feedback loop. In our own investigation, neuroimmune development was disturbed and may prematurely cause aberrant microglial responses, which would exacerbate the cascade of neuronal apoptosis and lack of proficient microglial responses.

We anticipate exploring the relationship of microglia with early neuronal degeneration in an AD model. Future studies will attempt to determine the exact developmental mechanism and sexually dimorphic immune vulnerabilities to AD by utilizing a wildtype murine model for comparison. Due to the early experimental time points and late pathological development, the terminal endpoints will be extended to ensure optimal visualization of any changes in pathologies. Similarly, immunofluorescent studies will attempt to illustrate the early involvement of microglia in aberrant neurogenesis through visualization of progenitor cell colonization. We anticipate these future studies will verify the seletive vulnerabilties in early neurimmune involvement in neurodegenerative diseases, as implied by the results of this study.

Figure 10. Immunofluorescent three color Z-stack imaging of a senile plaque in a treated male, PND 90. Microglia (green, FITC) are localized to the outer shell. Cell nuclei (blue, DAPI) show abnormal shape and clustering, most likely depicting dystrophic neurites. Amyloid-β (red, Cy5) accumulation can be seen near the inner core of the plaque.


50 µm

REFERENCES

Aguzzi, A., Barres, B.A., & Bennett, M.L. (2013). Microglia: scapegoat, saboteur, or something else? *Science, 339*, 156-161.

Baglioni, S., Casamenti, F., Bucciantini, M., Luheshi, L.M., Taddei, N., Chiti, F., Dobson, C.M., & Stefani, M. (2006). Prefibrillar amyloid aggregates could be generic toxins in higher organisms. *The Journal of Neuroscience, 26 (31)*, 8160-8167.

Basha, M.R., Wei, W., Bakheet, S.A., Benitez, N., Siddiqi, H.K., Ge, Y.W., Lahiri, D.K., & Zawia, N.H. (2005). The fetal basis of amyloidogenesis: exposure to lead and latent overexpression of amyloid precursor protein and β-amyloid in the aging brain. *The Journal of Neuroscience, 25(4)*, 823-829.

Bilbo, S.D. (2010). Early-life infection is a vulnerability factor for aging-related glial alterations and cognitive decline. *Neurobiology of Learning and Memory, 94 (1)*, 57-64.

Bolmont, T., Haiss, F., Eicke, D., Radde, R., Mathis, C.A., Klunk, W.E., Kohsaka, S., Jucker, M., & Calhoun, M.E. (2008). Dynamics of microglial/amyloid interaction indicate a role in plaque maintenance. *Neurobiology of Disease, 28 (16)*, 4283-4292.

Butchbach, M.E.R., Edwards, J.D., Schussler, K.R., & Burghes, A.H.M. (2007). A novel method for oral delivery of drug compounds to the neonatal SMNΔ7 mouse model of spinal muscular atrophy. *J Neurosci Methods, 161 (2)*, 285-290.

Combs, C.K., Karlo, J.C., Kao, S.C., & Landreth, G.E. (2001). B-Amyloid stimulation of microglia and monocytes results in TNFα-dependent expression of inducible nitric oxide synthase and neuronal apoptosis. *The Journal of Neuroscience, 21 (4)*, 1179-1188.

Cho, H., Hashimoto, T., Wong, E., Hori, Y., Wood, L.B., Zhao, L., Haigis, K.M., Hyman, B.T., & Irimia, D. (2013). Microfluidic chemotaxis platform for differentiating the roles of soluble and bound amyloid-β on microglial accumulation. *Scientific Reports, 3 (1823)*, 1-7.

Cras, P., Kawai, M., Siedlak, S., Mulvihill, Gambetti, P., Lowery, D., Gonzalez-DeWhitt, P., Greenberg, B., & Perry, G. (1990). Neuronal and microglial involvement in β-amyloid protein deposition in Alzheimer's disease. *American Journal of Pathology, 137 (2)*, 241-246.

DeWitt, J.C., Peden-Adams, M.M., Keil, D.E., & Dietert, R.R. (2012). Current status of developmental immunotoxicity: early-life patterns and testing. *Toxicologic Pathology, 40*, 230-236.

Dickson, D.W., Crystal, H.A., Bevona, C., Honer, W., Vincent, I., & Davies, P. (1995). Correlations of synaptic and pathological markers with cognition of the elderly. *Neurobiology of Aging, 16 (3)*, 285-304.

Dietert, R.R. (2011). Role of developmental immunotoxicity and immune dysfunction in chronic disease and cancer. *Reproductive Toxicology, 31*, 319-326.

Dietert, R.R., & DeWitt, J.C. (2010). *Immunotoxicity Testing: Methods and Protocols,* Methods in

Molecular Biology, 598, 17-25.

Donovan, M.H., Yazdani, U., Norris, R.D., Games, D., German, D.C., & Eisch, A.J. (2006). Decreased adult hippocampcal neurogenesis in the PDAPP mouse model of Alzheimer's disease. *The Journal of Comparative Neurology, 495*, 70-83.

Duncan, M.J., Smith, J.T., Franklin, K.M., Beckett, T.L., Murphy, M.P., St. Clair, D.K., Donohue, K.D., Striz, M., & O'Hara, B.F. (2012). Effects of aging and genotype on circadian rhythms, sleep, and clock gene expression in APPxPS1 knock-in mice, a model for Alzheimer's disease. *Experimental Neurology, 236*, 249-258.

Ford, A.L., Goodsall, A.L., Hickey, W.F., & Sedgwick, J.D. (1995). Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometry sorting. *The Journal of Immunology, 154*, 4309-4321.

Fiala, J.C. (2007). Mechanisms of amyloid plaque pathogenesis. *Acta Neuropathol, 114*, 551-571.

Graeber, M.B. & Streit, W.J. (2010). Microglia: biology and pathology. *Acta Neuropathol, 119*, 89-105.

Hagihara, H., Toyama, K., Yamasaki, N., & Miyakawa, T. (2009) Dissection of hippocampal dentate gyrus from adult mouse. *J Vis Exp, 33*, e1543.

Hickman, S.E., Allison, E.K., & Khoury, J.E. (2008). Microglial dysfunction and defective β-amyloid clearance pathways in aging Alzheimer's disease mice. *The Journal of Neuroscience, 28 (33)*, 8354-8360.

Hoozemans, J.J.M., Veerhuis, R., Van Haastert, E.S., Rozemuller, J.M., Baas, F., Eikelenboom, P., & Scheper, W. (2005). The unfolded protein response is activated in Alzheimer's disease. *Acta Neuropathol, 110*, 165-172.

Janelsins, M.C., Mastrangelo, M.A., Oddo, S., LaFerla, F.M., Federoff, H.J., & Bowers, W.J. (2005). Early correlation of microglial activation with enhanced tumor necrosis factor-alpha and monocyte chemoattractant protein-1 expression specifically within the entorhinal cortex of triple transgenic Alzheimer's disease mice. *Journal of Neuroinflammation, 2 (23)*, 1-12.

Karperien, A., Ahammer, H., & Jelinek, H.F. (2013). Quantitating the subtleties of microglial morphology with fractal analysis. *Frontiers in Cellular Neuroscience, 7 (3)*¸ 1-18.

Koenigsknecht, J. & Landreth, G. (2004). Microglial phagocytosis of fibrillar β-amyloid through a β1 integrin-dependent mechanism. *Neurobiology of Disease, 24 (44)*, 9838-9846.

Koo, J.W., Park, C.H., Choi, S.H., Kim, N.J., Kim, H.S., Choe, J.C., & Suh, Y.H. (2003). The postnatal environment can counteract prenatal effects on cognitive ability, cell proliferation, and synaptic protein expression. *The FASEB Journal, 17*, 1556-1559.

Lambert, M.P., Barlow, A.K., Chromy, B.A., Edwards, C., Freed, R., Liosatos, M., Morgan, T.E., Rozovsky,

I., Trommer, B., Viola, K.L., Wals, P., Zhang, C., Finch, C.E., Krafft, G.A., & Klein, W.L. (1998). Diffusible, nonfibrillar ligands derived from Aβ1-42 are potent central nervous system neurotoxins. *Proc Natl Acad Sci, 95*, 6448-6453.

Lazarov, O. & Marr, R.A. (2013). Of mice and men: neurogenesis, cognition and Alzheimer's disease. *Frontiers in Aging Neuroscience, 5*, 1-8.

Leifer, C.A., & Dietert, R.R. (2011). Early life environment and developmental immunotoxicity in inflammatory dysfunction and disease. *Toxicological & Environmental Chemistry*, 93:7, 1463-1485.

Lenz, K.M., Nugent, B.M., Haliyur, R., & McCarthy, M.M. (2013). Microglia are essential to masculinization of brain and behavior. *The Journal of Neuroscience, 33 (7)*, 2761-2772.

Lenz, K.M., Nugent, B.M., & McCarthy, M.M. (2012). Sexual differentiation of the rodent brain: dogma and beyond. *Frontiers in Neuroscience, 6 (26)*, 1-13.

Li, S., Hong, S., Shepardson, N.E., Walsh, D.M., Shankar, G.M., & Selkoe, D. (2009). Soluble oligomers of amyloid β protein facilitate hippocampal long-term depression by disrupting neuronal glutamate uptake. *Neuron, 62*, 788-801.

Lue, L.F., Kuo, Y.M., Beach, T., & Walker, D.G. (2010). Microglia activation and anti-inflammatory regulation in Alzheimer's disease. *Molecular Neurobiology, 41*, 115-128.

Mawuenyega, K.G., Sigurdson, W., Ovod, V., Munsell, L., Kasten, T., Morris, J.C., Yarasheski, K.E., & Bateman, R.J. (2010). Decreased clearance of CNS β-amyloid in Alzheimer's disease. *Science, 330*, 1774.

Moser, V.C., Walls, I., & Zoetis, T. (2005). Direct dosing of preweaning rodents in toxicity testing and research: deliberations of an ILSI RSI expert working group. *International Journal of Toxicity, 24,* 87-94.

Mu, Y. & Gage, F.H. (2011). Adult hippocampal neurogenesis and its role in Alzheimer's disease. *Molecular Neurodegeneration, 6 (85)*, 1-9.

Nikodemova, M. & Watters, J.J. (2012). Efficient isolation of live microglia with preserved phenotypes from adult mouse brain. *Journal of Neuroinflammation, 9 (147)*, 1-10.

Oddo, S., Caccamo, A., Kitazawa, M., Tseng, B.P., & LaFerla, F.M. (2003a). Amyloid deposition precedes tangle formation in a triple transgenic model of Alzheimer's disease. *Neurobiology of Aging, 24*, 1063-1070.

Oddo, S., Caccamo, A., Shepherd, J.D., Murphy, M.P., Golde, T.E., Kayed, R., Metherate, R., Mattson, M.P., Akbari, Y., LaFerla, F.M. (2003b). Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Aβ and synaptic dysfunction. *Neuron, 39*, 409-421.

Paolo, G.D., & Kim, T.W. (2011). Linking lipids to Alzheimer's disease: cholesterol and beyond. *Nat Rev Neurosci, 12(5)*, 284-296.

Paresce, D.M., Ghosh, R.N., & Maxfield, F.R. (1996). Microglial cells internalize aggregates of the Alzheimer's disease amyloid β-protein via a scavenger receptor. *Neuron, 17*, 553-565.

Parney, I.F., Waldron, J.S., & Parsa, A.T. (2009). Flow cytometry and in vitro analysis of human glioma-associated macrophages. *Journal of Neurosurgery, 110 (3)*, 572-582.

Pericak-Vance, M.A., & Haines, J.L. (1995). Genetic susceptibility to Alzheimer's disease. *Complex Diseases, 11*, 12, 504-508.

Pocock, J.M. & Kettenmann, H. (2007). Neurotransmitter receptors on microglia. *TRENDS in Neuroscience, 30(10)*, 527-535.

Portelius, E., Bogdanovic, N., Gustavsson, M.K., Volkmann, I., Brinkmalm, G., Zetterberg, H., Winblad, B., & Blennow, K. (2010). Mass spectrometric characterization of brain amyloid beta isoform signatures in familial and sporadic Alzheimer's disease. *Acta Neuropathol, 120*, 185-193.

Riascos, D., de Leon, D., Baker-Nigh, A., Nicholas, A., Yukhananov, R., Bu, J., Wu, C.K., & Geula, C. (2011). Age-related loss of calcium buffering and selective neuronal vulnerability in Alzheimer's disease. *Acta Neuropathol, 122*, 565-576.

Richartz, E., Batra, A., Simon, P., Wormstall, H., Bartels, M., Buchkremer, G., & Schott, K. (2005). Diminished production of proinflammatory cytokines in patients with Alzheimer's disease. *Dementia and Geriatric Diseases, 19*, 184-188.

Rodríguez, J.J., Jones, V.C., Tabuchi, M., Allan, S.M., Knight, E.M., LaFerla, F.M., Oddo, S., & Verkhratsky, A. (2008). Impaired adult neurogenesis in the dentate gyrus of a triple transgenic mouse model of Alzheimer's disease. *PLoS ONE, 3 (8)*, 1-8.

Schinder, A.F. & Morgenstern, N.A. (2009) Adult neurogenesis is altered by GABAergic imbalance in models of Alzheimer's disease. *Cell Stem Cell, 5*, 573-574.

Schmidt, R., Kienbacher, E., Benke, T., Dal-Bianco, P., Delazer, M., Ladurner, D., Jellinger, K., Marksteiner, J., Ransmayr, G., Schmidt, H., Stögmann, E., Friedrich, J., & Wehringer, C. (2008). Sex differences in Alzheimer's disease. *Neuropsychiatry, 22 (1)*, 1-15.

Schwarz, J.M., Sholar, P.W., and Bilbo, S.D. (2012). Sex differences in microglial colonization of the developing rat brain. *Journal of Neurochemistry, 120*, 948-963.

Sedgwick, J.D., Schwender, S., Imrich, H., Dörries, R., Butcher, G.W., & Meulen, V.T. (1991). Isolation and direct characterization of resident microglial cells from the normal and inflamed central nervous system. *Proc Natl Acad Sci, 88*, 7438-7442.

Selenica, M.L.B., Alvarez, J.A., Nash, K.R., Lee, D.C., Cao, C., Lin, X., Reid, P., Mouton, P.R., & Gordon, M.N. (2013). Diverse activation of microglia by chemokine (c-c motif) ligand 2 overexpression in brain. *Journal of Neuroinflammation, 10 (86)*, 1-17.

Stalder M., Phinney, A., Probst, A., Sommer, B., Staufenbiel, M., & Jucker, M. (1999). Association of microglia with amyloid plaques in brains of APP23 transgenic mice. *American Journal of Pathology, 154 (6)*, 1673-1683.

Stevens, S.L., Bao, J., Hollis, J., Lessov, N.S., Clark, W.M., & Stenzel-Poore, M.P. (2001). The use of flow cytometry to evaluate temporal changes in inflammatory cells following focal cerebral ischemia in mice. *Brain Research, 932*, 110-119.

Streit, W.J. (2010). Microglial activation and neuroinflammation in Alzheimer's disease: a critical examination of recent history. *Frontiers in Aging Neuroscience, 2 (22)*, 1-5.

Streit, W.J., Braak, H., Xue, Q.S., & Bechmann, I. (2009). Dystrophic (senescent) rather than activated microglial cells are associated with tau pathology and likely precede neurodegeneration in Alzheimer's disease. *Acta Neuropathol, 118*, 475-485.

Watt, A.D., Perez, K.A., Rembach, A., Sherrat, N.A., Hung, L.W., Johanssen, T., McLean, C.A., Kok, W.M., Hutton, C.A., Fodero-Tavoletti, M., Masters, C.L., Villemagne, V.L., & Barnham, K.J. (2013). Oligoers, fact or artefact? SDS-PAGE induces dimerization of β-amyloid in human brain samples. *Acta Neuropathol, 125*, 549-564.

Yuan, Y., Chen, Y.P.P., Boyd-Kirkup, J., Khaitovich, P., & Somel, M. (2012). Accelerated aging-related transcriptome changes in the female prefrontal cortex. *Aging Cell, 11 (5)*, 894-901.

Zoetis, T. & Walls, I. (2005). Principles and Practices for direct dosing of pre-weaning mammals in toxicity testing and research. A report of the ILSI Risk Science Institute Expert Working Group on Direct Dosing of Pre-weaning Mammals in Toxicity Testing, *ILSI Press, Washington DC.*

Zurich, M.G., Eskes, C., Honegger, P., Bérode, M., & Monnet-Tschudi, F. (2002). Maturation-dependent neurotoxicity of lead acetate in vitro: implications of glial reactions. *Journal of Neuroscience Research, 70*, 108-116.

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

May 6, 2013

Jamie DeWitt, Ph.D.
Department of Pharmacology
Brody 6S-10
ECU Brody School of Medicine

Dear Dr. DeWitt:

Your Animal Use Protocol entitled, "Exacerbation of Alzheimer's Pathology by Early-Life Exposure to Lead" (AUP #W236) was reviewed by this institution's Animal Care and Use Committee on 5/6/13. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

enclosure