The Pennsylvania State University
The Graduate School

NOVEL MODEL FOR LOADING BRAIN IRON IN MICE: IMPLICATIONS FOR STUDYING AGING AND AMYLOID PATHOLOGY

A Dissertation in

Neuroscience

by

Douglas Gordon Peters

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The dissertation of Douglas Gordon Peters was reviewed and approved by the following:

Mark D. Meadowcroft  
Assistant Professor of Radiology and Neurosurgery  
Dissertation Co-Advisor  
Co-Chair of Committee

James R. Connor  
University Distinguished Professor  
Vice Chair of Neurosurgery  
Dissertation Co-Advisor  
Co-Chair of Committee

Qing X Yang  
Professor of Radiology, Bioengineering  
Engineering Sciences and Neurosurgery  
Director, NMR Facility

Paul J. Eslinger  
Professor of Neurology, Neural & Behavioral Sciences, Pediatrics, Radiology,  
and Public Health Sciences  
Clinical Neuropsychology & Cognitive Neuroscience Programs  
Editor-in-Chief, Social Neuroscience

Ralph L. Keil  
Chair of Biomedical Sciences Graduate Program  
Associate Professor of Biochemistry and Molecular Biology

Colin J. Barnstable  
Professor and Chair of Neural and Behavioral Sciences  
Research Director of Penn State Hershey Eye Center

Signatures on file in the Graduate School
ABSTRACT

Brain iron accumulation occurs normally in the aging brain to facilitate myelination of white matter (WM); neurotransmitter synthesis; and ATP production. However, mishandling of iron may lead to early senescence and apoptosis because of impaired waste clearance, decreased cellular energy supply and increased oxidative stress. During the aging process, iron accumulates in regionally specific brain areas: basal ganglia, olfactory bulb, hippocampus, and cerebellum that are clinically associated with the manifestations of brain dysfunction in Alzheimer’s disease (AD) and neurodegeneration with brain iron accumulation (NBIA). Both conditions are linked to iron imbalance. Recent work illustrates that amyloid precursor protein (APP), a protein that is cleaved to form amyloid beta (Aβ), is important for iron clearance. Dysfunction of APP may impair normal iron balance, and iron imbalance may increase Aβ plaque genesis associated with AD.

This dissertation has explored the effect of dietary iron overload and deficiency in a wildtype control (C57BL6) and humanized APP knock-in mouse models (NL-F and NL-G-F), by using a small lipophilic iron molecule, TMHF. We found TMHF in both the blood and brain of mice after dietary exposure. Chronic TMHF exposure significantly increased brain iron in all animals by 15-35% within the hippocampus, cortex, basal ganglia, and cerebellum. Iron deficiency was only associated with decreased brain iron in the APP mutant mice. *In vivo* MRI analysis and immunohistological findings support the notion that MRI relaxation (R₂) is driven by iron deposition. TMHF sustained the MRI WM volume fraction that decreased over time in the normal brain iron condition.

WM loss with aging may propagate free iron increases, contributing to AD Aβ deposition. APP synthesis is a common marker for WM damage and is found to be elevated in
AD. This work found Aβ40 clustering and dense Aβ42 deposition near WM tracts. Although Aβ42 saturation is not altered by brain iron load, Aβ42 plaques was denser. In contrast, Aβ40 plaque load was shown to decrease over time with increasing brain iron accumulation. Increased Aβ42/40 ratio drives the proliferation of more hydrophobic oligomers that are believed to be more neurotoxic. The altered Aβ42/40 ratio found in the setting of iron accumulation may differentiate a primitive/diffuse plaque from a senile cored plaque. This altered ratio may also explain the accelerated process of iron accumulation and subsequent amyloid plaque deposition noted in AD, when compared to the normal aging process.

ELISA analysis illustrates that TMHF iron linearly increases the iron storage protein, L-Ferritin, to facilitate normal handling of elevated iron. Despite the normative response to sequester toxic free iron, TMHF also elevated the protein markers for oxidative stress, inflammation and gliosis associated with AD pathology. TMHF fed animals also developed impaired spatial learning as shown by Barnes maze testing.

With respect to iron dyshomeostasis, iron overload with TMHF increased Aβ plaque iron and iron-rich microglia. These iron inclusions may increase neurodegenerative processes during normal aging and disease. Using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) and Perl’s iron stain, we found regional heterogeneity of these iron deposits. The content of iron-rich plaques had a higher Aβ42/40 ratio and presented with more densely packed senile core-like features. IBA1+ microglia were also found to have shorter branches, rounder bodies, and intense iron staining in the TMHF group. An increased phagocytic profile during amyloid deposition with increased brain iron may perturb microglial housekeeping functions in the brain.
Taken together, we found that iron overload with TMHF increases brain iron in a physiologically normal and regionally specific manner. Brain iron elevation increases WM volume, but it may also increase inflammation, oxidative stress, and neurotoxicity of Aβ plaques, all of which are linked to memory impairment in normal aging and AD.
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<td>Aβ</td>
<td>Amyloid-Beta</td>
</tr>
<tr>
<td>AICD</td>
<td>Amyloid intracellular domain</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloproteinase domain-containing protein</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APOE</td>
<td>Apolipoprotein E</td>
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<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
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<tr>
<td>BACE-1</td>
<td>Beta-secretase 1</td>
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<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BCEC</td>
<td>Brain capillary endothelial cell</td>
</tr>
<tr>
<td>BCSFI</td>
<td>Brain cerebral spinal fluid interface</td>
</tr>
<tr>
<td>BCSFB</td>
<td>Blood cerebral spinal fluid barrier</td>
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<tr>
<td>CA1/2/3</td>
<td>Cornu ammonis area 1/2/3</td>
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<tr>
<td>CAA</td>
<td>Cerebral amyloid angiopathy</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CRP</td>
<td>C reactive protein</td>
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<td>Cerebral spinal fluid</td>
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<td>Cytochrome P450</td>
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<td>Duodenal cytochrome b</td>
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<tr>
<td>DFO</td>
<td>Deferoxamine</td>
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<td>DG</td>
<td>Dentate gyrus</td>
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<td>DMT1</td>
<td>Divalent metal transporter 1</td>
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<td>ECS</td>
<td>Extracellular space</td>
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<td>EOAD</td>
<td>Early onset Alzheimer’s Disease</td>
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<td>FAD</td>
<td>Familial Alzheimer’s Disease</td>
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<tr>
<td>FPN</td>
<td>Ferroportin</td>
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<td>FTL</td>
<td>Ferritin light chain</td>
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<td>FTH</td>
<td>Ferritin heavy chain</td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
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<tr>
<td>GD-DTPA</td>
<td>Gadolinium-diethylenetriamine pentaacetic acid</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein (astrocyte marker)</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
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<tr>
<td>GLUT1</td>
<td>Glucose transporter 1</td>
</tr>
<tr>
<td>GM</td>
<td>Gray matter</td>
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<td>GSH</td>
<td>Glutathione</td>
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<td>Hematocrit</td>
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<td>Hemoglobin</td>
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<td>HIF 1/2</td>
<td>Hypoxia inducible factor 1/2</td>
</tr>
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<td>HO-1</td>
<td>Hemoxygenase 1</td>
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<tr>
<td>H(U)PLC</td>
<td>High (Ultra)-performance liquid chromatography</td>
</tr>
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<td>HSPG</td>
<td>Heparan sulfate proteoglycan</td>
</tr>
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<td>HupA</td>
<td>Huperzine A</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional animal care and use committee</td>
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<tr>
<td>IBA-1</td>
<td>Ionized calcium-binding adapter molecule 1 (microglial marker)</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>IRE</td>
<td>Iron response element</td>
</tr>
<tr>
<td>IRP 1/2</td>
<td>Iron response element-binding proteins 1/2</td>
</tr>
<tr>
<td>LA-ICP-MS</td>
<td>Laser ablation inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>LDLR</td>
<td>Low density lipoprotein receptor</td>
</tr>
<tr>
<td>LRPI</td>
<td>Low density lipoprotein receptor-related protein 1</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCI</td>
<td>Mild cognitive impairment</td>
</tr>
<tr>
<td>MCV</td>
<td>Mean corpuscular volume</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MSME</td>
<td>Multi slice multi echo</td>
</tr>
<tr>
<td>NBM</td>
<td>Nucleus basalis of Meynert</td>
</tr>
<tr>
<td>NL-F</td>
<td>Swedish-Iberian APP mutation</td>
</tr>
<tr>
<td>NL-G-F</td>
<td>Swedish-Arctic-Iberian APP mutation</td>
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<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NrF2</td>
<td>Nuclear factor (erythroid-derived 2)-like 2</td>
</tr>
<tr>
<td>NTBI</td>
<td>Non-transferrin binding iron</td>
</tr>
<tr>
<td>OPC</td>
<td>Oligodendrocyte precursor cell</td>
</tr>
<tr>
<td>PIB</td>
<td>Pittsburgh compound B</td>
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<tr>
<td>PLT</td>
<td>Platelets</td>
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<tr>
<td>PSEN</td>
<td>Presenilin</td>
</tr>
<tr>
<td>pTau</td>
<td>Hyperphosphorylated Tau</td>
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<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>STEAP3</td>
<td>Six-transmembrane epithelial antigen of prostate 3</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>TE</td>
<td>Echo time</td>
</tr>
<tr>
<td>TfR1/2</td>
<td>Transferrin receptor 1/2</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>TIM2</td>
<td>Transmembrane immunoglobulin and mucin domain 2</td>
</tr>
<tr>
<td>TMHF</td>
<td>3,5,5 trimethylhexanoyl ferrocene</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TR</td>
<td>Repetition time</td>
</tr>
<tr>
<td>TREM 1/2</td>
<td>Triggering receptor expressed on myeloid cells 1/2</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cells</td>
</tr>
<tr>
<td>WM</td>
<td>White Matter</td>
</tr>
<tr>
<td>WT/C57BL6</td>
<td>Wild type mouse</td>
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</table>
ACKNOWLEDGMENT

I dedicate this thesis to my daughter, Juniper. She is a symbol of change—a reminder that the world is reborn every day. As she sees for the first time, eyes wide and attentive, we also see for the first time. My hope is that she can hold on to her wonder and innocence as I have tried.

I could not have done this without the love and support of my beautiful wife, Juliette. She brings me balance and relief when things are hard. She is my morning and evening bell.

To my parents, George and Anne, they instilled in me that education is the most honorable pursuit in life. I still remember my mother telling me on my first day of school, “you can never ask enough questions.” Perhaps, that is what has led me to this point. To my siblings, Danny, Bart and Katie, they are my best friends. I look forward to the weekly chats.

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Finally, I would like to thank the Leader and Harbolis Families for their generous financial contributions in support of my work and ongoing work in the lab.
EPIGRAPH

“Excessive reservations and paralyzing despondency have not helped the sciences to advance nor are they helping them to advance, but a healthy optimism that cheerfully searches for new ways to understand, as it is convinced that it will be possible to find them.”

- Alois Alzheimer
Chapter 1
The relationship between iron dyshomeostasis and amyloidogenesis in Alzheimer’s disease:
Two sides of the same coin

1.1 Abstract

The dysregulation of iron metabolism in Alzheimer’s disease (AD) is not accounted for in the current framework of the amyloid cascade hypothesis. Accumulating evidence suggests that impaired iron homeostasis is an early event in AD progression. Iron dyshomeostasis leads to a loss of function in several enzymes requiring iron as a cofactor, the formation of toxic oxidative species, and the elevated production of beta-amyloid proteins. Several common genetic polymorphisms that cause increased iron levels and dyshomeostasis have been associated with AD but the pathoetiology is not well understood. A full picture is necessary to explain how heterogeneous circumstances lead to iron loading and amyloid deposition. There is evidence to support a causative interplay between the concerted loss of iron homeostasis and amyloid plaque formation. We hypothesize that iron misregulation and beta-amyloid plaque pathology are synergistic in the process of neurodegeneration and ultimately cause a downward cascade of events that spiral into the manifestation of AD. In this introduction, we amalgamate recent findings of brain iron metabolism in healthy versus AD brains and consider unique mechanisms of iron transport in different brain cells as well as how disturbances in iron regulation lead to disease etiology and propagate Alzheimer’s pathology.
1.2 Introduction

AD is the most common cause of dementia affecting six million people in the United States and forty million people worldwide. The literature supports that there are two distinct clinical manifestations of AD; familial and sporadic. Both are characterized by molecular lesions attributed to the aggregation of misfolded proteins, inflammation and metabolic failure leading to neurological dysfunction. Familial AD (FAD), also characterized as Early Onset AD (EOAD), has been linked to genetic mutations, affects people under 60 years old, but makes up less than 5% of all Alzheimer’s cases. Sporadic Alzheimer’s, also referred to as Late Onset (LOAD), is the most common form affecting people over 60 and has no direct pattern of inheritance. Although AD is not a certain outcome of the aging process, age is the primary risk factor as the incidence for LOAD doubles every five years after 65. The prevalence of AD is expected to triple by 2050 as the average lifespan increases in the future.

The recognition that iron dyshomeostasis is critical in AD pathology is based on observations that patients have elevated iron levels in cortical, subcortical, and WM areas affected by the disease. MRI analysis reveals that increased iron levels in the hippocampus, an important structure perturbed early in AD, negatively correlates with memory test performance. Increased iron loading in the brain is also associated with beta-amyloid (Aβ) plaque formation, where it is focally incorporated into the core and halo regions, and hyperphosphorylated tau (pTau) tangles in the brain. Increasing the concentration of iron in vitro accelerates Aβ plaque and pTau tangle aggregation and increases their toxicity. Iron dyshomeostasis may lead to toxic pathological features, but the same imbalance can disrupt innate biological systems that depend on iron.
Iron is one of the most abundant elements on earth and was utilized by early organisms before our current oxygen rich atmosphere was established\(^{11}\). As a transition metal, iron is uniquely involved in reductive and oxidative (redox) cycling reactions and as a cofactor in iron-sulfur clusters within numerous enzymes\(^{12}\). The majority of the body’s iron (70\%) is bound to hemoglobin within red blood cells to aid in tissue oxygen transport. The balance of non-hemoglobin bound iron is found within proteins (~6\%) facilitating the metabolic energy needs of the body through cellular respiration (ATP synthesis through the TCA cycle, ferredoxin, cytochromes, and aconitase) and those involved in ribosome function, DNA repair, and synthesis\(^{13-15}\). The remainder is stored within globular ferritin protein complexes (~24\%) for controlled iron sequestration, detoxification, and release\(^{16}\). Proper iron maintenance is critical for the body and in the brain; thus, there are specialized molecules, cells, regions, and organs for storing and releasing iron.

The brain has a large amount of iron, unevenly distributed to the neurons of the basal ganglia, brain capillary endothelial cells (BCECs), and glia. The basal ganglia requires iron for neurotransmitter synthesis, BCECs confer an iron shuttle between the blood and brain, and astrocytes and microglia help to distribute and sequester iron in the perenchyma\(^{17-19}\). The relative distribution of non-heme iron in the vertebrate body is 55\% liver, 20\% kidney, 15\% heart, and 10\% brain\(^{20}\). During vertebrate brain development the brain is highly permeable to iron to facilitate neural growth and intercellular connection\(^{21}\). After development, the brain tightly controls circulating non-heme iron entering and exiting the brain. The brain normally acquires approximately 10\% of its iron from the diet where it crosses the gut and enters the blood stream, relatively less iron than any other organ: three-fold less than the liver\(^{20}\). Thus, the brain has more reserve non-heme iron than any other organ to carry out its function. The reason
behind this is not well understood, but there are several possibilities. Firstly; the brain is one of the most metabolically active organs in the body, consuming a significant amount of the body’s oxygen\textsuperscript{22}. This requires the brain to have an iron reserve to assure that its energy requirements are met during a potential lull in iron status. Secondly; iron influx into and efflux out of the brain is tightly controlled by the blood brain barrier (BBB), brain cerebrospinal fluid interface (BCSFI), and the blood CSF barrier (BCSFB)\textsuperscript{18,23,24}. This regulation resides outside the control of the brain making it difficult for the brain to finely adjust the influx and efflux of iron. Thirdly; while neuronal growth and division in the brain during adulthood is minimal, limiting the necessity for new exogenous iron to create new synaptic connections, iron rich oligodendrocytes continually require large amounts of the element\textsuperscript{25}. 

Brain iron is most prevalent in oligodendrocytes where it is required in the myelination of neuronal axons to form the WM in the brain facilitating saltatory conduction over longer distances with increased speed\textsuperscript{26}. Humans have proportionally more white to gray matter than any other animal, and we are the only species to have heterochronologic development with brain regions myelinating at different time points\textsuperscript{27,28}. This difference may help to explain our advanced cognitive processes and IQ as well as human brain atrophy later in life\textsuperscript{29,30}. Iron deficiency during infancy causes delayed neurocognitive development, impaired learning and memory, and in some cases, psychiatric disorders; all in part due to the impairment of myelination\textsuperscript{31}. Oligodendrocyte precursor cells (OPCs) propagate throughout life to form new oligodendrocytes to myelinate additional structures and to replenish damaged myelin during remyelination. As the brain ages, remyelination is outpaced by myelin breakdown leading to a loss of WM volume\textsuperscript{28}. The rate and loss of WM due to normal aging increases free iron that is toxic to the brain. \textit{In vivo} magnetic resonance imaging (MRI) analysis illustrates that WM
damage precedes gray matter damage in AD compared to age-matched controls\textsuperscript{32}. Exacerbated WM loss late in life can contribute to increased neuronal loss associated with AD. This damage can be due to increased toxic radical production and impaired molecular systems necessary to ameliorate them.

Alterations in iron load and the proteins responsible for iron metabolism can exacerbate the excess formation and harmful effects of reactive oxygen (ROS) and nitrogen species (RNS), leading to cell death\textsuperscript{33}. The increased expression of amyloid precursor protein (APP) at the site of axonal damage may underlie the need for amyloid proteins to repair tissue and manage cellular debris\textsuperscript{34}. The response of amyloid and tau to free radicals and hypoxia induced ischemia, as is witnessed in brain trauma, is hypothesized to ameliorate their effects. However, over time APP and cleaved amyloid fragments harbor ferric iron (Fe\textsuperscript{3+}) that cannot be stored or transported adequately\textsuperscript{35,36}. Aβ bound Fe\textsuperscript{3+} is easily reduced to ferrous iron (Fe\textsuperscript{2+}), escalating the production of ROS species\textsuperscript{37}. This milieu favors increased pathogenic production of toxic Aβ oligomers and plaques that propagate from monomeric Aβ\textsubscript{42} through β-secretase cleavage\textsuperscript{38,39}. Once formed, tau tangles and aberrant amyloid structures induce synaptic loss and apoptosis independent of ROS\textsuperscript{40,41}.

Dietary ingredients that chelate iron and remove oxidative species are hypothesized to reduce the onset time of AD\textsuperscript{42–44}. Deferoxamine (DFO), a ferric iron chelator used to treat iron overload, decreases tau phosphorylation, slows down amyloidogenesis, and improves behavioral impairment in an Alzheimer’s mouse and rabbit models\textsuperscript{45–48}. DFO also improves behavioral performance in AD patients after a 24 month administration period\textsuperscript{49}. However, the longitudinal follow-up of these studies have not been adequately performed. Huperzine A (HupA), a natural inhibitor of acetylcholinesterase, is a licensed anti-AD drug in China that may possess other
neuro-protective properties that are not well understood. Recent work shows that treatment with HupA reduces iron in the brain that correlates with a reduction in insoluble and soluble Aβ, amyloid plaque formation, and hyperphosphorylated tau in the cortex and hippocampus of an AD mouse model. Increasing dietary iron negates any benefit from HupA, suggesting that elevated iron is involved in AD pathoetiology\textsuperscript{50}.

The relationship between iron, amyloidogenesis, and AD is multi-faceted. There are four essential questions that are important to understand how iron is associated with AD. 1) Is iron dyshomeostasis a primary or a secondary factor of AD pathoetiology? 2) Is the brain iron imbalance in AD caused by a dysfunctional or an overwhelmed iron regulatory system? 3) Is brain iron dyshomeostasis derived from peripheral iron or is it coming from endogenous brain iron? 4) How are iron, amyloidosis, and cognition related; is the etiology of AD one of Aβ plaque formation, iron dyshomeostasis, or a synergy of both processes?

1.3 The Amyloid Cascade Hypothesis

Over the last twenty years the amyloid cascade hypothesis has helped scientists unearth a slew of critical genomic and proteomic data to better characterize amyloid plaque formation as a pathological marker of AD and the mechanism behind amyloidosis. Parallel to this, researchers have accumulated data on iron level regulation in the peripheral and central nervous systems. The developing metallostasis field has emphasized the role of improper metal ion redox cycling during aging and how the improper balance of metals in the brain may lead to amyloid deposition, cell dysfunction, and apoptosis found in AD\textsuperscript{51}. Iron is not the only transition metal that undergoes dyshomeostasis in the aging and diseased brain state. Copper (Cu) and zinc (Zn) play an important physiological function in the brain, however their concentrations are much lower than that of iron\textsuperscript{52}. The role of APP and pathogenic Aβ derivatives as metalloproteins
highlight their complex relationship with iron loading and metabolism in AD\textsuperscript{53,54}. Current findings indicate that metallostasis should be included within the framework of the amyloid cascade hypothesis.

Although the association of APP to AD has been thoroughly studied there remain a number of uncertainties such as its relationship with iron transport. APP is processed by several enzymes producing smaller fragments that participate in normal cellular function in an unknown fashion. APP is cleaved by α-secretase (ADAM) or β-secretase (BACE-1) producing the extracellular soluble fragments sAPP\textalpha or sAPP\textbeta, respectively. The alpha pathway is a non-amyloidogenic process while the beta pathway is amyloidogenic. The membrane bound C-terminal fragments, C83 or C99 produced during α and β cleavage, are cleaved by γ-secretase to generate extracellular fragment p3 or \(\text{Aβ}_{40/42}\), respectively. The remaining C-terminal fragment, amyloid intracellular domain (AICD), is released into the cytoplasm after progressive cleavage by the γ-secretase/presenilin (PSEN) complex, where it is targeted to the nucleus to act as a transcription factor. Increased production of \(\text{Aβ}_{42}\) leads to the formation of insoluble extracellular amyloid fibrils and amyloid plaques. APP and secretase mutations were initially discovered to be related to FAD because they lead to increased production and aggregation of \(\text{Aβ}_{42}\). Transgenic mice containing these same mutations express similar hallmark pathological features and memory deficits. The transgenic pathology, however, does not completely mimic that found in human disease; potentially due to lower brain iron or APP translational regulation\textsuperscript{55,56}. Transgenic plaques formed in mice do not contain the same amount of focal iron and do not bind Pittsburgh Compound B (PIB), a marker for fibrous insoluble Aβ plaques\textsuperscript{7,57}. Profound Aβ plaque and pTau tangle formations in non-human vertebrates do not lead to the memory deficit.
observed in AD pathology. This may be due to reduced plaque toxicity in non-human Aβ plaques which do not bind iron\(^58\).

Fe\(^{2+}\) and Fe\(^{3+}\) interactions with APP and Aβ facilitates the extent and speed of Aβ aggregation into fibrillar forms\(^10,59\). Seeded plaques can sequester other metals (Zn, Cu, Al, Mn) and a variety of cellular products, such as lipids, impairing proper cellular function\(^60,61\). Aβ\(_{42}\) is increased with APP expression or BACE-1 activity or decreased ADAM activity. The Aβ\(_{42/40}\) ratio is dependent on γ-secretase function. Aβ\(_{42}\) and Aβ\(_{40}\) can both interact with iron; however, the Aβ\(_{42}\) fragment forms amyloid aggregates more rapidly. The Alzheimer’s brain shifts from a non-amyloidogenic ADAM/Aβ\(_{40}\) state to an amyloidogenic BACE-1/ Aβ\(_{42}\) state. Aggregation of Aβ\(_{42}\) initially follows a reversible linear progression of monomers, dimers, misfolded globules, soluble oligomers, seed β-sheet intermediates, protofibrils, fibrils, and senile fibril plaques (Fig. 1-1). Fe\(^{3+}\) has the capacity to bind all amyloid formations but has difficulty disassociating from the late fibrillar forms. The seeding of Aβ\(_{42}\) with insoluble Fe\(^{3+}\) may allow the initial clearance of excess free iron in the brain through plaque formation and microglial lysosomal degradation. However, some of that Fe\(^{3+}\) is lost by reduction to Fe\(^{2+}\) facilitated by its interaction with Aβ\(_{42}\)\(^62\). Fe\(^{2+}\) has also been shown to interact with Aβ amino acids, and this may confer changes to amyloid formations in a different manner than Cu and Zn\(^63,64\). When enough amyloid deposition has occurred, toxic oligomeric formations can propagate in a nonlinear amyloidogenic positive feedback-loop, bypassing the requirement for amyloid monomers to form dimers\(^39\). It is currently unknown how iron is involved in nonlinear Aβ plaque formation. The mature amyloid plaque core contains magnetite in the form of Fe\(^{2+}\) and Fe\(^{3+}\)\(^65\). The hydrophobic nature of the plaque core is believed to occlude electron sharing within the region, while more easily accessible iron in the outer halo could feasibly be a site of increased redox cycling.
Amyloid plaque formation can be characterized by primary and secondary aggregation. During primary formation, ferric iron interacts with beta amyloid as it fluctuates between monomer, dimer, and tetramer formations. Iron-amyloid interactions increase ferrous iron, which can lead to the production of reactive oxygen species. Chronic interaction with iron leads to misfolded globules that can form beta-sheet intermediates and toxic soluble oligomers in the secondary aggregation phase. At a certain threshold of amyloid fibril formation, monomers can propagate the production of toxic soluble, bypassing the primary amyloid formation in a non-linear aggregation phase. Iron can form coordinated covalent bonds with metal ions. Magnetite iron (Fe$^{2+}$ and Fe$^{3+}$) is believed to become entombed within the dense mature fibril plaque core with hindered redox activity. The diffuse halo of protofibril Aβ 40/42 around the core contains ferric and ferrous iron and it is hypothesized that iron in this region is more susceptible to reductive-oxidative cycling. The mature amyloid plaque serves to deplete ferric iron necessary for brain function and propagate ferrous iron that leads to oxidative stress.
Knock-out removal of endogenous APP in mice increases neuronal iron, while overexpression of the Aβ containing APP c-terminal fragment reduces neuronal iron\textsuperscript{66,67}. In addition, \textit{in vivo} iron overload in the APP/PS1 AD mouse model leads to increased APP processing, altered neuronal signaling, and cognitive decline with increased amyloid plaque formation\textsuperscript{68}. This suggests that brain iron load is directly tied to the initial stages of amyloid processing and plaque formation. While there are findings that suggest total brain iron content is not altered in the AD brain, regional analysis provides evidence that harmful macroscopic focal deposition of iron is occurring in and around amyloid plaques\textsuperscript{67,69,70}. Illustrating this is the knowledge that \textit{in vivo} MRI imaging of the AD brain demonstrates increased iron in the hippocampus; one of the central brain regions afflicted by amyloidogenesis\textsuperscript{4}.

\textbf{1.4 Brain Iron Accumulation with Aging}

Iron accumulates in the brain over time, reaching an apparent plateau between thirty and forty years of age\textsuperscript{71}. While the work of Hallgren and Sourander demonstrates the plateauing of brain iron levels, it is evident that the distribution between individuals becomes more variable after the age of 60\textsuperscript{71}. A relative decline may reflect a brain that is decreasing its iron absorption or increasing its release. A relative increase illustrates a brain that is either increasing its iron absorption and/or decreasing its iron release. Increasing brain iron may be due to several factors including, but not limited to, the imbalance in iron transport and storage proteins, mitochondrial dysfunction, neurovascular mechanisms, and myelin breakdown and disrepair\textsuperscript{72-74}. Late onset AD presents after age 65 that chronologically coincides with an increase in brain iron in subjects prone to iron overloading\textsuperscript{75} (Fig. 1-2). While AD is generally considered an aging disease it is of interest that early onset AD presents itself as early as 40 years of age, coinciding with the plateauing of iron levels in the brain\textsuperscript{76}. This trend also is apparent in Down’s syndrome where a
Figure 1-2

Graph showing the relationship between age, brain iron, and relative cognition. The graph illustrates the impact of normal mental decline, high iron (High Fe), low iron (Low Fe), early onset AD, and late onset AD on brain iron levels across different ages.
Figure 1-2. The hypothesized inverse relationship of brain iron elevation and relative cognitive decline in AD and an aging population

The mean brain iron concentration (black line) sharply increases early in life with little population variance. In midlife brain iron concentration plateaus but population variance expands\textsuperscript{71,238}. Normal mental decline for an aged individual decreases slowly over time. The incidence of Early Onset AD and Late Onset AD correlate with increased iron and variability in the brain\textsuperscript{239–241}. 
trisomy of chromosome 21 results in APP overexpression, a six-fold likelihood of developing amyloid plaque pathology, and brain iron deposition at 35 years old\textsuperscript{77}.

1.5 Oxidative Stress and Toxic Mechanisms

The free radical theory of aging postulates that reactions with metal generated oxygen free radicals result in a pattern of increased cellular aging\textsuperscript{78}. Brain aging involves structural, functional, and chemical changes that occur over time within normal health and neurodegenerative disease. Current research suggests that oxidative stress contributes to brain aging by means of altered DNA, enhanced cell death, lipid peroxidation, protein dysfunction, decreased degradation, and altered metabolism\textsuperscript{79,80}.

Iron facilitates the energy needs of the growing and highly active human brain. Most physiological iron is stored and utilized as insoluble Fe\textsuperscript{3+} and iron homeostasis and cytoplasmic transportation are dependent on soluble Fe\textsuperscript{2+}. Unregulated iron homeostasis leads to an increased Fe\textsuperscript{2+}/Fe\textsuperscript{3+} ratio which is harmful to neuronal cells as Fe\textsuperscript{2+} drives oxidative stress through Fenton and Haber-Weiss chemistry\textsuperscript{81}. In addition, the relative decrease of Fe\textsuperscript{3+} is indirectly harmful as it promotes the reduction of superoxide radicals derived from mitochondrial activity\textsuperscript{82,83}. Consequently, excess superoxide may react with nitric oxide (NO) to produce other toxic species.

NO is produced from nitric oxide synthase (NOS) and neuronal damage caused by excessive NO is termed nitrosative stress\textsuperscript{84}. NO is a highly diffusible gas produced by neurons, endothelial cells, and glial cells to optimize blood flow by enhancing vasodilation and VEGF production, as well as synaptic excitability\textsuperscript{85–87}. Inhibition of NO increases APP expression, BACE-1 activity, and Aβ production\textsuperscript{88}. NO levels are increased in AD brain tissue and are hypothesized to retard amyloidogenic pathway progression. The apparent increase in NO to
combat amyloid production is a double-edged sword as increased levels of NO are harmful to neural cells. Increased NO levels react with superoxide and produce peroxynitrate, which impedes tyrosine residue function\(^9^9\). In AD, nitration of tau proteins prevents them from stabilizing the microtubule lattice and nitrated tau has been observed in tau tangles and amyloid plaques\(^9^0,9^1\). Inflammation and excessive neuronal activation by glutamate can induce NOS, which increases the production of NO\(^8^4\). In addition, NO disrupts the iron-sulfur core within aconitase, altering the production of iron regulatory proteins. Therapies that can mitigate oxidative stress and inflammation may help mediate the progress of AD. It has been shown that vitamin E and Ibuprofen reduce amyloid and improve memory deficits\(^9^2,9^3\). The effect of these therapies may augment the innate antioxidant and inflammatory mechanisms that are disturbed in AD pathology.

Healthy neuronal cells have ROS and RNS detection and degradation pathways that buffer toxic molecular species. Nuclear factor E2-related factor 2 (Nrf2) affects the homeostasis of ROS and RNS by initiating the antioxidant response element (ARE) pathway, resulting in catabolism of superoxide and peroxides, regeneration of oxidized cofactors and proteins, synthesis of reducing factors, expression of antioxidants, and metal chelation. Nrf2 is post-transcriptionally activated by several chemicals, including NO\(^9^4\). In AD transcription of Nrf2 is impaired and overexpression in an AD mouse model improves spatial memory\(^9^5,9^6\).

Glutathione (GSH) is an important thiol-based antioxidant found in the brain that is primarily produced by astrocytes. GSH plays a prominent role in the detoxification of ROS in the aging brain and is reduced in AD astrocytes and microglia cells. Furthermore, AD associated GSH depletion is observed in the inferior frontal and inferior temporal cortices; primarily areas damaged in AD\(^9^7\). Cultured astrocytes, microglia, and oligodendrocytes exposed to brief
hypoxia express less GSH, reduced redox capacity, and apoptosis; partially explaining the
decrease in AD cortical GSH\textsuperscript{98,99}. Brain iron levels are related to damage induced by GSH
depletion as the chelation of iron with DFO blocks cellular death in a reduced GSH milieu\textsuperscript{100}.

1.6 Iron Storage and Transport in the Brain

The homeostatic balance of cellular iron in the brain is orchestrated through six distinct processes: transport, uptake, storage, utilization, redox cycling, and export. The bulk of iron transportation throughout the body is performed by the glycoprotein transferrin (Tf)\textsuperscript{101}. Oligodendrocytes and choroid plexus cells are the only two neural cell types that produce Tf. Tf expression is decreased in AD within WM and stains diffusely in and around the halo region of cortical Aβ plaques, suggesting that the AD brain has an impairment in this tissue\textsuperscript{5}. It is unknown if the localized iron within the Aβ plaques offers a binding site for Tf where upon it becomes encapsulated within the plaque mass. In addition to its normal distribution within oligodendrocytes, Tf is also found abnormally associated within astrocytes of the AD brain which suggests a homeostatic iron imbalance in these cells\textsuperscript{9}. The exact role of astrocytes in this regard is speculated to be related to the sequestration of oligodendrocyte Tf during demyelination or through the phagocytosis of amyloid with bound Tf.

Brain iron uptake is generally regulated by transferrin receptor 1 (TfR1) and divalent metal transporter 1 (DMT1)\textsuperscript{102,103}. TfR1 is highly expressed on arterial endothelial cells at the BBB and choroid plexus epithelial cells at the BCSFB\textsuperscript{104}. Expression on the luminal side is tightly controlled, decreasing when brain iron is replete or increasing when brain iron is depleted. TfR1 is also expressed on the surface of neuronal cells and reactive microglia, facilitating neuronal iron import and microglial iron sequestration during inflammation. Transferrin receptor expression is reduced in the AD hippocampus, a region heavily affected by early
amyloidopathy. The dentate gyrus (DG) of the hippocampus is a distinct region of the brain where neurogenesis occurs and newly divided cells highly express TfR1. The cause for the formation of large punctate Aβ plaques in the AD DG has not been thoroughly investigated but is hypothesized to be related to the inability of this region to limit its iron intake and a progressive increase in iron content. Iron import during non-Tf-bound-iron (NTBI) transport is facilitated by DMT1, whose gene sequence (SLC11A2) is located adjacent to regions commonly mutated in AD on the long arm of chromosome 12. In addition, inflammatory cytokines increase the expression of DMT1 within neurons, astrocytes, and microglia in the AD brain.

Cellular iron storage, detoxification, and necessary release is regulated by ferritin, a 450 kDa protein complex consisting of 24 subunits of light (L-) and heavy (H-) ferritin. H-Ferritin serves as a ferroxidase, producing ferric iron within the core of the ferritin protein. L-ferritin seeds the quaternary structure with H-ferritin to hold up to 4500 ferric iron ions depending on intracellular iron levels. Ferritin distribution during normal aging remains constant throughout the brain. The majority of ferritin is found heterogeneously in oligodendrocytes, but it is also present in neurons, protoplasmic astrocytes, and microglia. In vivo MRI measures reveals that basal ganglia ferritin is greater in younger LOAD patients compared to controls, with the difference being absent in older late onset AD patients. The AD brain presents with robust microglial ferritin around amyloid plaques and blood vessels. Elevated levels of CSF ferritin are apparent in AD and it is unclear if ferritin enters the CSF from the blood or brain parenchyma. It is known that CSF ferritin increases after a subarachnoid haemorrhage and can be produced intrathecally following a bleed.

Iron is utilized by mitochondrial proteins, such as frataxin, mitoNEET, and numerous other enzymes in the brain that require iron to act as an electron carrier. Deficiency of
Frataxin is noted in Friedreich’s ataxia, the disease in which it was first described. While the physiological function of frataxin is unclear, it is believed to act as an iron chaperone in the assembly of iron-sulfur clusters for which neurons have high demand. In addition, an extra-mitochondrial frataxin was recently discovered to stabilize the Fe-S cluster in aconitase offering molecular control of cytosolic aconitase/IRP1. MitoNEET is an integral iron containing protein in the outer leaflet of mitochondria. It is implicated in moderating the maximal respiratory rate by controlling the amount of iron imported into the mitochondria and controls oxidative species produced by mitochondrial respiration. Reduction of mitoNEET leads to enhanced mitochondrial respiratory capacity through increased matrix incorporation of iron and elevated oxidative stress. Damage or dysfunction of frataxin and mitoNeet is speculated to be involved in several facets of AD including ROS control, redox control, and heme metabolism; however, no studies have directly connected mitoNeet and frataxin dysfunction to amyloid production.

The reduction and oxidative (redox) cycling of iron is required to meet cellular energy demands through which ROS are generated. Careful compartmentalization during redox cycling by neural proteins facilitates detoxification and shuttling of iron throughout the brain. Extracellular and intracellular H-ferritin detoxifies and stores iron by oxidizing soluble Fe$^{2+}$ to insoluble Fe$^{3+}$. Ferrous iron is oxidized by ceruloplasmin and hephaestin and is subsequently effluxed as Fe$^{2+}$ by means of ferroportin and Tf. Reduction of Fe$^{3+}$ by an endosomal STEAP3 and peripheral membrane cellular prion protein (PrPc) facilitates the influx of iron by means of TfR and NTBI. PrPc is present at its highest levels in neurons of the brain, but it is also present at lower levels in neural glial cells. Binding of Aβ to PrPc impairs ferrireductase
activity and the loss of PrPc results in the iron overloading of the brain paired with elevated Tf and TfR1 transcription, as discussed in detail in the next section\textsuperscript{121,122}.

Export of iron is predominantly facilitated by the transmembrane protein ferroportin. Ferroportin transports ferrous intracellular iron in tandem with ferroxidases, such as ceruloplasmin, to oxidize and transport ferric iron into the extracellular domain. It is hypothesized that a region within the E2 domain of sAPP stabilizes ferroportin at the cell surface and leads to increased iron efflux\textsuperscript{123,124}. It is unknown how this function differs before and after APP cleavage by alpha and beta secretase. Once extracellular, apo-transferrin binds ferric iron and is shuttled to a transferrin receptor on a nearby neuron or it is hypothesized to be carried out of the brain via binding to transferrin receptors on the apical surface of an arterial endothelial cell of the BBB or the basal surface of an ependymal cell at the BCSFI. Ferric iron can also be reduced and imported into the cell via NTBI transport. If iron is not oxidized in the ferroportin core, the hormone hepcidin binds to ferroportin and triggers the internalization and degradation of the receptor-ligand complex\textsuperscript{125}. Hepcidin is mostly secreted by the liver to control peripheral iron levels, but neural protein expression is elevated on activated astrocytes and microglia exposed to inflammatory cytokines\textsuperscript{108,126}. Ferroportin reduction in an AD mouse model is associated with heme granular deposits adjacent to damaged blood vessels suggesting that vascular dysfunction is altering iron flow and homeostasis in the AD brain\textsuperscript{127}.

1.7 Iron protein transcription and translation

Iron balance in all cell types is regulated by the transcription of hypoxia-inducible factor (HIF) 1 and 2. HIF1 and HIF2 are structurally similar, but they have unique glycolytic control, and are expressed in discrete cell types in the brain\textsuperscript{128–130}. Both HIF1 and HIF2 consist of a cytosolic subunit HIFα and the constitutively expressed nuclear subunit HIFβ. The HIF
heterodimer forms in low iron, low oxygen, or high NO condition to produce target RNA transcripts with iron regulatory elements (IREs), including TfR1 and DMT1. HIFα degradation is mediated by the ubiquitin-proteasome system when iron and oxygen are available. Ischemic events, iron loading, or disrupted proteasomal function alters HIF function and lowers TfR1 and DMT1 levels in the AD brain. PSEN1/PSEN2 modulates the functional response to hypoxia by altering HIFα expression\textsuperscript{131}. Stabilizing HIF with a prolyl-hydroxylase inhibitor in normal aging mice enhances hippocampal memory, providing a potential therapy for AD\textsuperscript{132}. Iron chelators, such as M30, upregulate HIF and HIF-activating compounds in cortical neurons\textsuperscript{133}.

Iron regulatory protein 1 (IRP1) or IRP2 bind to hairpin IREs present in the untranslated regions (UTRs) of protein mRNA that are important for iron storage and transport\textsuperscript{134}. IRP1 functions as an aconitase in a high Fe, low O$_2$, or low NO environment. In opposite conditions, IRP1 binds a UTR IRE at either the 5’ or 3’ position of the pre-translated RNA transcripts. When the UTR IRE is in the 5’ position, RNA polymerase is blocked from translating the target protein; as is the case for H-/L-ferritin, ferroportin, APP, HIFα, and PrPc. If the UTR IRE is in the 3’ position, IRPs stabilize the mRNA and facilitate the translation of the target protein such as TfR1 and DMT1. IRP2 acts similarly to IRP1, except that it is inactivated via ubiquitination under sufficient iron conditions. IRP has an increased affinity to IRE in the AD brain explaining the elevation of iron levels without a rise in ferritin production\textsuperscript{135}. The mechanism behind this stability is not understood and is speculated to be related to the rise in endogenous ribonuclease activity observed in AD\textsuperscript{135}.

1.8 Brain Perfusion

AD and iron dyshomeostasis are hypothesized to correlate to a disruption of brain vasculature. Micro-casting of AD mouse model brains reveals that there are alterations of the
microvasculature accompanied by Aβ attachment and deposition. Aged transgenic mouse brain vessels are occluded and terminate at Aβ plaques, causing small gaps in the micro-vasculature net around which angiogenesis is occurring\textsuperscript{136}. While the relationship between AD, iron homeostasis, and cerebral amyloid angiopathy (CAA) is not fully understood, we hypothesize that brain perfusion and iron dyshomeostasis are related to amyloid generation.

CAA results in the deposition of Aβ in the meningeal and intracerebral vessels. CAA presents in over 50% of elderly individuals and is also found in 50% of AD cases highlighting that it is a common occurrence in the aging brain but does not always associate with AD\textsuperscript{137}. There is a distinction of iron loading in AD with and without CAA; tissue from AD patients with severe CAA had significantly higher non-heme iron levels especially in the walls of large vessels\textsuperscript{138}.

It is hypothesized that improper clearance of Aβ and the buildup of iron at the BBB can lead to aberrant angiogenesis and senescence of the cerebrovascular system\textsuperscript{139}. These environmental conditions begin a process of neurovascular uncoupling, vessel regression with vascular smooth muscle loss, brain hypoperfusion, and neurovascular inflammation. Ultimately, these conditions lead to BBB leakage, improper drainage, chemical imbalance in the neuronal environment, and synaptic dysfunction and loss\textsuperscript{73}. CAA and iron deposition lead to the degradation of cytoskeletal elements and aquaporin-4 channels localized on astrocytic end feet at the BBB\textsuperscript{140}. There is also a concomitant loss of glucose transporter-1 and lactate transporter in these astrocytes\textsuperscript{141}. Further research needs to be conducted to illustrate if the initial ischemia and deposition of Aβ and iron at the cerebral wall is dependent on brain disease or peripheral dysfunction.
The source of amyloid deposition is heterogeneous; concussion and hemorrhage increase the risk of developing Aβ in the brain. Aβ can bind to red blood cell (RBC) heme, a process that increases with aging, and incubation of erythrocytes with Aβ decreases NOS synthesis, causing deformity and increased cellular volume. It is unclear how RBC Aβ levels correlate with CAA; however, RBC dysfunction helps explain the hypoperfusion and heme deposition at occluded vessels in AD.

Recent findings indicate that a paravascular pathway exists in the brain that facilitates CSF flow through the brain parenchyma and allows clearance of interstitial solutes and Aβ. This glymphatic system is dependent on aquaporin-4, arterial pulsation, and is enhanced during sleep. It is unclear if impairment of this process in AD leads to poor Aβ clearance or if CAA and iron deposition precede these alterations.

1.9 Neuron

Neurons display TfR1 more than any other cell type in the brain as its expression mediates iron import into these cells (Fig. 1-3). Iron bound Tf has a high affinity for TfR1 and is regulated through competitive binding with HFE, a major histocompatibility complex class I-like molecule. HFE mutations (H63D and C282Y) increase Tf/TfR1 binding and lead to hemochromatosis and oxidative stress in the brain. H63D neurons express higher APP and Aβ, and in vitro Aβ induces apoptosis via mitochondrial dysfunction and caspase activation. The knowledge that 29.7% of the Caucasian population are carriers for HFE mutations highlights the importance of this polymorphism. Transferrin (C2) and HFE mutations, leading to iron overload, are also strongly correlated to AD. Any known population benefit for these mutations is not well understood, but recent findings suggest that Tf mutations provide a means to counteract bacterial iron piracy in primates during infection.
Figure 1-3
Figure 1-3. The neuron in AD

A) Tf binds to TfR1 with augmentation through HFE interaction. Clathrin coat mediated endocytosis occurs, releasing ferrous iron into the cytoplasm via STEAP-3 reductase and DMT1 present in the endosome. Tf and TfR1 are recycled back the cell surface for further Tf binding\textsuperscript{242}. B) Under healthy conditions soluble tau traffics APP to the membrane where it acts to stabilize ferroportin for iron export\textsuperscript{67,124}. C) PrPc increases NTBI uptake by acting as a ferrireductase and increasing ferrous iron near the exoplasmic cell membrane\textsuperscript{118}. In AD PrPC binding to amyloid oligomers disinhibits tau expression via the FYN pathway leading to increased APP expression at the cell surface\textsuperscript{121}. FYN activation also causes reduced NMDA expression, which leads to synapse dysfunction and cell death\textsuperscript{121}. D) Increased cell membrane cholesterol causes elevated β-secretase activity, which promotes Aβ\textsubscript{42} levels along the amyloidogenic pathway\textsuperscript{168}. E) Mitochondrial proteins help to store and reduce excessive oxidation from the TCA cycle\textsuperscript{115}. Mitochondrial dysfunction is hypothesized to increase the labile iron pool\textsuperscript{243}. F) High extracellular iron leads to the influx of iron into the neuron via Ca\textsuperscript{2+} channels, leading to toxicity\textsuperscript{176}. G) Ischemia leads to hypoxia mediated HIF and IRP alterations; TfR1 expression is elevated and ferroportin expression is decreased leading to increased iron intake and ROS/RNS production\textsuperscript{99,244}. H) Iron induced pTau aggregation throughout the neuron leads to microtubule destabilization and Aβ toxicity\textsuperscript{40,245}. 
When TfR1 forms a complex with Tf, it is taken into the cell via clathrin-mediated endocytosis (Fig. 1-3A). Within the endosome, Fe$^{3+}$ is released from Tf upon acidification and STEAP3 reduces the iron to Fe$^{2+}$ for export from the endosome into the cytoplasm by DMT1. The endosome is recycled back to the cell surface to redeposit HFE, TfR1, and DMT1. Ferroportin shuttles ferrous iron into its channel and works in tandem with ferrooxidases, such as ceruloplasmin, to shuttle ferric iron out. Released ferric iron can bind to Tf or it can be reduced and taken back into the cell via NTBI transport. It is hypothesized that Aβ modifies this process through ferric iron interaction preventing binding with Tf, hindering iron transport$^{154}$.

Soluble tau is necessary to shuttle APP to the neuronal surface so that it can interact with ferroportin iron export (Fig. 1-3B). Blocking soluble tau results in neuronal iron overload, as does knocking out APP$^{155,156}$. In neuronal cell cultures Fe$^{3+}$ induced hyperphosphorylated tau (pTau) aggregation and Fe$^{2+}$ generated oxidative radicals can induce tau hyperphosphorylation via activation of the extracellular signal regulated kinase1/2 (Erk1/2) pathway or the mitogen-activated protein kinase pathway (MAPK)$^{157–159}$. In AD, increased iron co-localizes with insoluble tau tangles and facilitates the loss of soluble tau, further perturbing tau-mediate APP shuttling and ferroportin iron export$^{142,155}$. The usage of iron chelators highlights this relationship as they decrease the phosphorylation of tau and its aggregation$^{45}$. The loss of soluble tau prevents tau stabilization of microtubules, altering molecular transport important for neurotransmission, synapse stability, and cellular health$^{160}$.

Phosphorylated tau tangles develop in the axon hillock, soma, and axon of neurons (Fig. 1-3H). Tau aggregates can be transferred to adjacent cells and downstream cells synaptically connected in a prion-like manner$^{161}$. This spread leads to sequential cellular degeneration at the synapse, axon, and soma$^{162}$. This agrees with the finding that the presence of pTau at the
synapse increased the synaptic toxicity of amyloid oligomers. There are two potential synergistic mechanisms by which iron-amyloid-induced degeneration is related to pTau pathology: 1) amyloid cannot sequester all of the extraneuronal iron, leading to increased transport of intraneuronal iron via NTBI, increased phosphorylation of tau, and neuronal dysfunction; or 2) elevated intraneuronal iron increases APP processing and the level of extracellular amyloid oligomers, consequentially leading to amyloid-pTau toxicity at the synapse. In addition to inter-neuronal cytoskeletal perturbations, alterations in the cholesterol dynamics modify the cellular membrane fluidity.

AD is positively correlated with increased serum cholesterol\textsuperscript{163}. Cholesterol metabolites traveling in the blood from the liver elevate cholesterol levels in the brain, clarifying the interaction between serum cholesterol and \textit{de novo} brain synthesis. Aβ levels are decreased in an AD mouse model that synthesizes 50\% less sterol. Conversely, induced hypercholesteremia in a rabbit animal model fed a cholesterol rich diet increases amyloidosis and cortical distribution of iron\textsuperscript{164}. As neuronal cholesterol increases, the fluidity of the membrane is reduced and lipid raft composition is altered, decreasing α-secretase contact with APP\textsuperscript{165}. Low cholesterol is linked with increased α-secretase activity and decreased production of toxic Aβ oligomers\textsuperscript{166,167}. There is evidence that β-secretase translation is upregulated by intracellular cholesterol and downregulated by cholesterol synthesis inhibition\textsuperscript{168}. In addition, γ-secretase activity is dependent on cholesterol found in lipid rafts. Removing cholesterol prevents γ-secretase activity and Aβ production\textsuperscript{169}. CNS cholesterol synthesis is also linked to the cerebral accumulation of pTau\textsuperscript{170}. Taken together, increasing cholesterol pushes the brain into an AD amyloidogenic state (Fig. 1-3D). The process becomes more complex when observing the effects of cholesterol synthesis byproducts.
Brain derived 24S-hydroxycholesterol downregulates the β-cleavage of APP, decreases cholesterol synthesis, and upregulates APOE, aiding in the efflux of excess cholesterol from the brain. Peripheral 27S-hydroxycholesterol from the liver crosses the BBB and may be linked to hypercholesteremia in the brain where upon APP and Aβ metabolize cholesterol to oxysterol 7-beta hydroxycholesterol, a neurotoxic byproduct that contributes to oxidative stress\textsuperscript{171}.

PrPc regulates iron influx through ferrireductase activity at the cell surface and interaction with DMT1 and the Zip Fe/Zn transporter to shuttle ferrous iron across the cellular plasma membrane independently of TfR\textsuperscript{172}. PrPc also facilitates normal cell function by modulating molecular pathways, such as stress inducible factor 1, inducing growth, and synaptic function. The binding of PrPc to amyloid oligomers present in the AD brain increases tau expression to help shuttle more APP to the cell surface; decreasing neuronal iron while increasing Aβ production. This adversely affects neurons as NMDAR expression is decreased when PrPc is exposed to amyloid oligomers\textsuperscript{173} (Fig. 1-3C). The depletion of NMDAR is subsequently followed by synaptic loss and cell death via the FYN pathway\textsuperscript{121}. The misfolding of PrPc into PrP-scrapie in Cruetzfeldt-Jakob disease leads to iron dyshomeostasis and elevated PrP\textsuperscript{Sc} has been found in the AD brain\textsuperscript{122,174}.

Long-lasting L-Type voltage gated calcium channels are localized to the soma and dendrites of neurons and glial cells, coupling membrane depolarization in neurons to gene expression, synaptic efficacy, and cell survival. Activation leads to a primary influx of calcium and secondary transport of iron through these channels when extracellular iron increases (Fig. 1-3F). This process leads to neuronal iron overloading and toxicity\textsuperscript{175,176}. The same channels facilitate iron protection or iron burden depending on the cell type and iron overloading status\textsuperscript{176,177}. 
Iron regulatory protein production is tightly regulated by iron and oxygen concentration. Decreased oxygen levels increases TfR and DMT1 expression and decreased ferroportin production, leading to elevated iron loading within the cell (Fig. 1-3G). Increased NO may be a compensatory mechanism to improve blood flow in ischemia, underlining vascular pathology as a potential shift in iron homeostasis. NO synthase activity is increased in AD leading to amplified free radical production as well as NO that in turn, disrupts IRP function and reduces iron export.  

1.10 Astrocyte  

Astrocytes function within the neuronal network to bridge cells and connect them to the vasculature and ventricular systems (Fig. 1-4). Astrocyte glia limitans end-foot processes surround endothelial cells to create the BBB and regulate traffic into the brain parenchyma. The small interstitial space separating the glia limitans and endothelial cells must be traversed to allow penetration into the brain. Under healthy conditions, astrocytes acquire iron across the interstitial space using distinct NTBI mechanisms such as citrate, ATP, ascorbic acid, DMT1, and Zip (Fig. 1-4A). High iron conditions stimulate astrocytes to become reactive where upon they become enlarged with cellular processes. Astrocytes strongly express ferritin upon stimulation with iron and are highly resistant to iron toxicity. They convey a neuroprotective role through the intake of excess extracellular iron via the upregulation of ferritin synthesis, DMT1, L-type Ca²⁺ channels, transient receptor potential (TRP) channels, ATP secretion, endocytosis of debris, and downregulation of ferroportin; allowing astrocytes to act as an iron sink. Astrocytes increase APP and β-secretase expression under high iron conditions, illustrating that these cells are similar to neurons in how they utilize APP and Aβ to regulated excess iron. Under healthy conditions, astrocytic iron is believed to be released from ferritin
Figure 1-4
Figure 1-4: The astrocyte in AD

A) Astrocytes acquire iron from NTBI sources, including citrate, ATP and ascorbic acid\textsuperscript{179}. B) Astrocytes are scavenger cells, facilitating the cleanup of high extracellular iron concentrations via Zip, DMT1, VGCC and TRP channels. These importers become more active or upregulated during high extracellular iron conditions in AD\textsuperscript{180}. C) The astrocyte-neuron shuttle facilitates a lactate energy source for neurons under high activity. This connection is weakened in AD by astrogliosis and microtubule pTau interference\textsuperscript{186}. D) MitoNEET is an iron-containing protein that is an insulin sensor\textsuperscript{117}. Insulin is sensed by the astrocyte, causing it to increase glycogen stores and lactate production. Insulin sensitivity is reduced in AD leading to neuronal dysfunction\textsuperscript{246}. E) Astrocytes are a major source of APOE with increased export when exposed to Aβ\textsuperscript{198}. Haptoglobin secretion is elevated during astrogliosis, facilitating the binding of Aβ to APOE\textsuperscript{206}. F) LRP-1, LDLR, and HSPG facilitate APOE endocytosis. Aβ binds to these receptors and blocks APOE endocytosis and metabolism\textsuperscript{207}. The extracellular increase in APOE is hypothesized to be a source of Aβ seeding. G) Astrogliosis protects oligodendrocytes exposed to Aβ by reducing ROS or removing Aβ through endocytosis\textsuperscript{247}. H) Astrocytes produce the antioxidant glutathione and highly express ferritin. They are known to store high levels of iron while being resilient to ROS/RNS\textsuperscript{248}. Decreased glutathione and ferroportin make astrocytes susceptible to iron toxicity in AD and the aging process. I) The ferroxidase ceruloplasmin is primarily expressed by astrocytes which promotes the influx of NTBI. Its activity is decreased in AD leading to an increase in the extracellular ferrous iron pool\textsuperscript{249}. J) The astrocytic endfoot serves to regulate iron import into the healthy brain for which damage to these processes in AD lead to unregulated brain iron import\textsuperscript{141}. 
via lysosomal degradation and the resulting free iron is exported with ferroportin. Evidence suggests that iron efflux from astrocytes plays a role in remyelination through a direct interaction with oligodendrocyte precursor cells or indirectly by activating microglial cells. However, when astrocytes fail to return to a resting state in AD they diminish their metabolic support.

Astrocytes are the only cells in the brain that facilitate glycogen and glutamate synthase, which is utilized for glucose metabolism by hypoglycemic neurons and oligodendrocytes. Astrocyte-neuron lactate shuttling is shown to be critical for long-term memory formation that becomes weak in AD, preventing the sharing of lactate (Fig. 1-4C). Blood insulin levels play a role in astrocyte function as in vitro exposure to insulin promotes glycogen storage and cell proliferation in primary human astrocytes. Evidence suggests insulin plays a role in amyloidosis as high levels of physiological insulin infusion increase APP and Aβ42 production.

Patients in early stages of type 2 diabetes show brain dysfunction while diabetic patients 55 and older on the insulin sensitizer metformin were less likely to acquire dementia. The mechanism by which this occurs is hypothesized to be related to either hyperinsulinemia upregulation of BACE-1 and APP, decreased Aβ degradation activity of insulin degrading enzyme (IDE), or decreased mitochondria mitoNEET activity (Fig. 1-4D).

Apolipoprotein E (APOE), predominantly produced by astrocytes, has been identified as an associated factor with late onset AD. APOE’s primary role is that of a cholesterol transporter, however it also regulates Aβ transport, metabolism, aggregation, and deposition. APOE has three major polymorphisms in humans: ε2 (8%), ε3 (77%), and ε4 (15%). ε4 is significantly associated with the onset and severity of AD with a prevalence in 40% of AD patients. ε2 has been shown to be protective against AD, however it is a risk factor for cardiovascular disease. APOE binds Aβ in an isoform-dependent manner, ε2> ε3> ε4, and
facilitates clearance of Aβ by either lysosomal degradation within astrocytes or shuttling Aβ out of the brain via the BBB or BCSF1203–205. Under non-amyloidogenic conditions, APOE enables the clearance of Aβ by either facilitating its degradation in the brain or shuttling it out of the brain via the BBB or BCSF1. APOE binds LRP1, LDLR, and HSPG present on neurons, vascular endothelial cells and neurons. These receptors are responsible for lipid metabolism and are present in all major cellular pathways that clear Aβ206. Aβ not complexed with APOE blocks these receptors, inhibiting APOE metabolism and transport207 (Fig. 1-4F).

During amyloidosis APOE seeds amyloid deposition independent of fibrilogenesis in an ε4>ε3 manner and it is unknown how metals interact within this process. There is a strong positive correlation between APOE and CSF ferritin levels in ε4 carriers and patients with low APOE or high ferritin in the CSF showed greater brain atrophy208. It is hypothesized that APOE functions to lower neuronal iron as APOE KO mice exhibit elevated brain iron208. Cells overexpressing APOE have less ferritin and WT cells exposed to iron increased APOE expression208. APOE attenuates Aβ induced astrogliosis in cultured cells, but it is unknown how astrogliosis alters APOE levels209. Reactive astrocytes elevate the expression of haptoglobin, and increases binding of Aβ with APOE210 (Fig. 1-4E). However, under stress conditions, APOE becomes fragmented resulting in increased Aβ deposition, mitochondrial, and cytoskeletal damage. Decreasing APOE has been shown to reduce amyloid plaque formation in AD mouse models, but the importance of APOE for cholesterol homeostasis and as a Aβ/Fe shuttle may make it harmful as a long-term treatment211.

1.11 Oligodendrocyte

Oligodendrocytes are unique to the brain as they are the only cell with intracellularly synthesized Tf, they express the Tim2 ferritin transporter, and they contain a large amount of the
brain’s lipids and cholesterol (Fig. 1-5). In addition, oligodendrocytes contain the majority of
the healthy brain’s iron in the core of ferritin complexes. High concentrations of iron are
required during myelination and levels must be tightly controlled to maintain iron homeostasis.
As demyelination and myelin breakdown outpace remyelination during normal aging, ferritin
iron and free iron collect in the extracellular space. In the healthy brain, proper iron management
by other neural cells eliminates excessive iron toxicity in this domain. Normal brain WM
atrophies and loses 20% of its volume from age 30 to 80, while gray matter atrophies less than
6%. MR imaging of the AD brain reveals that excessive brain atrophy is initiated by myelin
degradation followed by GM atrophy; forming the hypothesis that increased myelin loss may
contribute to subsequent GM atrophy. The volume of WM lesions is inversely related to
proper APOE function in middle-aged and elderly subjects, suggesting that impaired cholesterol
metabolism and oligodendrocyte damage are related. Oligodendrocytes serve as the brain’s
primary pool of cholesterol and lipids. Lipidation byproducts in the blood are a biomarker for
the onset of memory impairment in AD. This is most likely due to the loss of plasma
membrane from myelin and neuronal synapsis. Impaired lipid and cholesterol metabolism can
also lead to hypercholesterolemia, which was earlier described as being harmful in AD.

The mechanism of oligodendrocytes Tim2 ferritin receptor endocytosis is unlike neuronal
Tf endocytosis as ferritin is not recycled back into the extracellular space (Fig. 1-5A). Tim2-
bound ferritin endocytosis is followed by lysosomal degradation and elevated endogenous
ferritin synthesis. Tim2 acts as the iron uptake pathway for oligodendrocytes and is integral to
oligodendrocyte health and iron regulation. As Aβ fragments in vitro are observed to be
cytotoxic to oligodendrocytes in a dose-dependent manner, damage to oligodendrocytes during
Figure 1-5. The oligodendrocyte in AD

A) Tim2 facilitates ferritin endocytosis and iron release via lysosomal degradation\textsuperscript{217}. B) Normal breakdown and dysfunction of myelin with age releases iron and impairs trophic support\textsuperscript{250}. Apoptotic oligodendrocytes release ferritin into the extracellular space. Loss of WM can reduce ferritin storage in AD. C) Transferrin serves to transport iron precisely to metabolic regions of myelination. AD oligodendrocytes contain lower transferrin levels and have reduced myelination and remyelination\textsuperscript{9}. D) Oligodendrocytes express APP and can secrete Aβ\textsubscript{42}\textsuperscript{219}. Production and exposure to these proteins are heightened in demyelination and remyelination pathways. E) Loss of WM in AD leads to neuronal cognitive decline and cell death\textsuperscript{74}. F) It is likely that ROS from oligodendrocytes can lead to tau destabilization of microtubule mediated axonal trafficking, disrupting axonal transport towards and from synaptic terminals. G) Exosome release from damaged oligodendrocytes activate microglia to cause further demyelination\textsuperscript{226}. 
AD may impair the clearance of ferritin in the brain and leave most of the iron storage to astrocytes\textsuperscript{218}.

Oligodendrocytes uniquely synthesize Tf with no known secretion mechanism. While it is unclear why oligodendrocytes synthesize Tf, the current hypothesis suggests that Tf may be necessary to transport Fe along the long stretches of myelin wrappings to and from the soma. Decreased Tf in AD can impair oligodendrocytes from properly transporting iron\textsuperscript{9} (Fig. 1-5C). Similar to astrocytes, oligodendrocytes express APP to aid in iron export or as an iron binding agent during bouts of iron overload. Increased APP expression in WM during brain trauma may well indicate the proteins role in the sequestration of iron during myelin damage. The high expression of APP also illustrates the cells role as a potential source for Aβ aggregation\textsuperscript{219}. Myelin breakdown can expose APP to β-secretase on neighboring neurons, facilitating the amyloidogenic process (Fig. 1-5D).

1.12 Microglia

Quiescent microglia in the brain contain highly branched ramified processes in their resting state (Fig. 1-6). Inflammatory molecules cause microglia to shrink into an amoeboid form that serves as the brain’s macrophage. Microglia respond to a number of molecular signals and are attracted via Aβ and ATP chemotaxis. As such, amyloidogenesis and astrogliosis mediate microglial movement toward a sites of localized inflammation\textsuperscript{220}.

Microglia recognize Aβ via CD36-α6β1-CD47 leading to the phagocytosis of iron-bound Aβ aggregates, lysosomal degradation, and the production of ROS. Active microglia surrounding large Aβ plaques synthesize elevated levels of ferritin to form a ferritin laden border along the microglial cell membrane\textsuperscript{221}. Continued intake and degredation of Aβ and iron bound to Aβ leads to increased levels of ROS and RNS with uncontrolled iron uptake. As AD
Figure 1-6
Figure 1-6. The microglia in AD

A) Microglia are drawn to the site of amyloidosis and astrogliosis via NTBI ATP and Aβ chemotaxis\textsuperscript{251}. B) Microglia recognize Aβ via the CD36-CD47 receptor system leading to the phagocytosis Aβ fibrils and bound iron\textsuperscript{252}. C) Amyloid is normally degraded via the lysosomal pathway and ferrous iron is stored in ferritin\textsuperscript{224}. In the AD state, excess iron and amyloid overwhelm the lysosome and the decreased pH provides a suitable environment for amyloid aggregation. D) ROS/RNS produced from redox cycling of iron impairs the lysosomal breakdown of Aβ and promotes cleavage and neurotoxicity of tau\textsuperscript{224,253}. E) Ferritin can be secreted from microglia through a non-classical secretory pathway. This process is impaired in AD, leading to an imbalance of iron transport to oligodendrocytes. F) Microvesicles secreted from activate microglia interact with soluble Aβ to form amyloid oligomers that cause oligodendrotoxicity and neurotoxicity\textsuperscript{225}. G) Macropinocytosis of exosomes produced by damaged oligodendrocytes cause microglia to facilitate myelin degradation and inflammation\textsuperscript{226}. H) Hypoxic conditions increase IRPs and TfR expression, leading to an increase in ROS and further cytokine release.
progresses in the brain, Aβ and ferritin iron overwhelm and slow the efficiency of the microglial lysosome. In addition, the acidic environment of the microglial lysosome has been suggested to favor Aβ aggregation. Improper degradation of ferritin in the microglial lysosome is hypothesized to lead to the accumulation of toxic hemosiderin and magnetite deposits, which cannot be metabolized (Fig. 1-6D).

Small homogenous microvesicles (exosomes) are shed by all neural cell types via the release of multivesicular bodies along the endocytic pathway, serving as a means of exoplasmic communication between cells (Fig. 1-6F). Microglial microvesicles interact with soluble Aβ and initiate the formation of Aβ oligomers that are toxic to synapses. Macropinocytosis of exosomes produced by damaged oligodendrocytes cause microglia to release inflammatory cytokines (Fig. 1-6G). CSF microvesicle concentration is correlated with WM damage in MCI and with hippocampal atrophy in late onset AD. This follows the trend of WM damage prior to gray matter atrophy in AD, as previously described. It has been suggested that hippocampal microglia activation in the presence of excess Aβ favors the formation of toxic Aβ oligomers that progress brain-wide via WM damage and microglial activation. Hypoxic conditions during AD increase IRP activity and TfR expression of microglial, leading to continued ROS and RNS production and further inflammatory cytokine release (Fig. 1-6H).

1.13 Discussion – The Big Picture

The integration of iron dyshomeostasis and amyloidogenesis denotes that concomitant features of both synergistically amalgamate along related paths towards Alzheimer’s pathology (Figs. 1-7 and 1-8). Microcapillary lesions contribute to a wide array of problems that may contribute to the AD process and propagate increased extracellular iron. Proper BBB regulation is no longer maintained allowing hemoglobin, transferrin, ferritin, and free iron from the blood to
Figure 1-7
Figure 1-7. A systemic illustration of cellular iron cycling and amyloid production in the healthy brain

A) Blood and transferrin bound iron metabolites flow through a microcapillary vessel network. Transferrin receptor is highly expressed on the luminal surface of the vessel endothelial cells and binds to holo-transferrin. Tranferrin is transcytosed or endocytosed into the endothelial cell. Ferric iron is released into the interstitial space where it is temporarily bound to astrocyte secreted NTBI factors. It is then reduced by ferrireductases present on astrocyte endfoot processes and imported into the astrocyte via DMT1. B) Ferric iron is packed into astrocyte ferritin for temporary storage. C) Ferritin is broken down in a late endosome/lysosome to restore ferrous iron into the labile iron pool. D) ROS are minimized by the endogenous antioxidant glutathione. E) Ferrous iron is transported down astrocyte processes, oxidized to its ferric form by ceruloplasmin, and effluxed via ferroportin. F) Transferrin binds to ferric iron and is transported into neurons via clatherin-mediated endocytosis. G) Normal neuronal iron causes the post-translational expression of APP to occur via IRP1 inactivity. H) APP is cleaved by α-secretase and γ-secretase to produce non-amyloidogenic fragments. β-secretase activity is low due to low cell membrane cholesterol and extracellular iron levels. I) APOE is produced from the astrocyte rough endoplasmic reticulum and exported via a secretory pathway. J) Amyloid fragments bound to iron complex with APOE and are endocytosed and degraded in the astrocytic or microglial lysosome. K) Microglia import iron via transferrin endocytosis and export iron via ferroportin and ferritin exocytosis. L) Extracellular ferritin and ferric iron can be endocytosed via oligodendrocyte expressed Tim2 or NTBI import. Oligodendrocytes stabilize axons to provide insulation for action potentials and facilitate myelination-remyelination throughout life.
M) Iron exits the brain through the ventricular CSF via transferrin, NTBI signaling, or APOE amyloid complexes.
Figure 1-8

[Diagram of cellular interactions, possibly involving neurons, astrocytes, oligodendrocytes, and microglia.]
Figure 1-8. A systemic illustration of cellular iron cycling and amyloid production in the AD brain

A) Microvascular events in the Alzheimer’s brain cause the deposition of Aβ along the luminal surface. Amyloid deposition occludes vessels and impairs the blood brain barrier allowing red blood cells to penetrate into the parenchyma, causing heme deposits to form near vessel walls. Aβ in the blood stream binds to and deforms circulating red blood cells causing impaired oxygen saturation. B) ROS present at the BBB cause astrocytes to withdraw their endfoot processes while secreting NTBI molecules and upregulating ferrous iron transporters. C) The increased influx of ferrous iron in astrocytes upregulate ferritin formation for intracellular iron storage. D) Glutathione is reduced in AD, exaggerating the effects of deleterious ROS. Activated astrocytes increase their iron load while expressing cytoplasmic APP. E) APOE secretion is elevated facilitating the sequestration of Aβ42 in the extracellular space. F) High extracellular iron concentration causes neurons to continually influx iron via NTBI and neuronal iron import. G) Neuronal iron export is enhanced through increased post-translational expression of APP which acts to export iron via ferroportin. H) Elevated cholesterol in the neuronal cell membrane alters secretase activity to elevate the production of Aβ42 production. I) Iron efflux from neuron helps seed Aβ42 progressing into Aβ fibrils and plaques. Microglia migrate toward activated astrocytes and Aβ deposits where they phagocytose Aβ plaques. J) Lysosomal degradation of Aβ plaques increase iron within the microglia which is in turn is incorporated into ferritin along their cytoplasmic border. Glutathione levels are decreased and the ferritin degradation and secretion pathway is impaired. Hemosiderin deposits form inside microglia further impairing their function. K) Reduction of ferritin export impairs oligodendrocytes iron uptake reducing remyelination. L) In addition, microvesicles communicated between microglia and
oligodendrocytes cause myelinotoxicity and increased microglial inflammatory cytokine release. 

M) Demyelination sloughs off high amounts of toxic iron, lipids and cholesterol into the exoplasmic space. This process also exposes APP present in the myelin sheath, which can propagate amyloidosis. 

N) There is a reciprocal increase of iron proteins and ferrous iron in the CSF and a decrease of amyloid products in the CSF, which may reflect a failure of amyloid clearance out of parenchymal space.
enter the brain contributing to iron overload. Cerebrovascular breakdown is linked to several factors including impaired metabolic transport across the BBB, reactive microglia bypassing the glial sheath, endothelial degeneration, and endfoot separation. Astrocytic endfeet normally contain a high concentration of mitochondria and surround the endothelial vascular tube forming the perivascular endfoot membrane. Disruption of the BBB in AD correlates with impaired GLUT1 and lactate transporter functionality as well as astrocyte endfoot withdrawal and astrogliosis; all of which precede Aβ deposition. BBB leakage progresses with age, especially with APOE deficiency compounded with brain injury. Astrocytes help distribute iron throughout the brain and clear excess extracellular iron. AD astrocytes abnormally store increased amounts of ferritin, APP, and Aβ intracellularly. It is unclear if this process contributes to plaque formation or if it is related to the failure of astrocyte or microglial lysosomal and iron export processes.

We hypothesize that amyloid production increases to ameliorate iron overload and patch the damaged vascular system. The time course of this progression is congruent with the plateauing of brain iron levels as early onset AD first presents itself during this period. The continued heterogeneity of iron levels in the aged population past this plateau and the incidence of iron incorporation in amyloid plaques further provide indication of a plausible interaction between these processes (Fig. 1-2). Regions that have relatively lower concentrations of iron, such as the frontal lobe and temporal lobes, form amyloid pathology. Regions with high iron content, such as the brainstem, motor cortex and cerebellum, are not affected by amyloidosis. We hypothesize that low iron brain regions are more susceptible to changes in iron homeostasis, elevated free iron, and subsequent amyloidosis compared to endogenous high iron regions that are maybe better equipped to maintain iron equilibrium. Increased free neuronal iron upregulates
APP translation and, when paired with aberrant secretase activity, leads to increased extracellular Aβ42, tau phosphorylation, neuronal toxicity, dysfunction, destabilization, and intraneuronal tau tangle formations.

Genetic mutations may cause a patient subset to be less suited to manage iron overload while other mutations may prompt patients to follow an amyloidogenic path in a high iron milieu. The relationship between these two circumstances represent a cross-road for Alzheimer’s progression. The higher incidence of AD in women is also of interest and cannot purely be attributed to increased female longevity. Women are more susceptible to the APOE ε4 variant and female AD mouse models present greater amyloid deposition than their male counterparts231,232. Our iron-amyloid hypothesis of AD helps to explain, in part, why this may be the case. The amount and density of long-term iron stored in the male (600 mg) body within ferritin is double that of women (300 mg). This suggests that the useable iron reservoir in women is in constant flux between intake and redox cycling. We hypothesize that females utilize short-term iron storage for immediate or intermediate usage compared to men (eg Tf or NTBI). Iron in this state may be more susceptible to interactions with Aβ and APOE leading to the genesis of toxic amyloid species.

The pathogenesis of AD is heterogeneous with numerous physiological features correlated to its progression. Brain iron overload does not always lead to amyloidogenesis and AD and, conversely, amyloidogenesis does not always lead to iron overload233. Current literature supports the hypothesis that there is a link between iron and amyloid dyshomeostasis in the AD brain. However, the exact nature of this relationship remains unclear and encompasses multiple possibilities; the aging AD brain may absorb more iron, transport iron improperly, or insufficiently release iron. Further distinction between cell culture, animal model, and human
studies require assessment due to differential endogenous iron levels, variations in oxygen saturation, and contributions from various cell types and pathologies making translation between these systems difficult. The finding that amyloid plaque burden does not correlate well to memory loss in patients suggests that there is a plaque-independent mechanism that is responsible for AD memory impairment.

Therapies aimed at reducing amyloid have proved problematic in clinical trials as the removal of amyloid from the brain releases free iron and destabilizes microvessels. Targeted reduction of Aβ deposition and amyloidogenesis with secretase inhibitors and immunotherapies leads to iron accumulation and cognitive deficits. These outcomes are not observed in AD model mice perhaps due to lower endogenous iron levels in mice than found in the human brain or translational modification of humanized sequences. The data support the hypothesis that amyloid in the brain serves as part of an iron maintenance mechanism and is initially protective against iron-mediated damage. A better understanding of the time course of iron dyshomeostasis and AD onset are needed to help guide therapies that can stabilize iron levels before they can become harmful in at-risk populations. Iron chelating agents, such as clioquinol, given at an appropriate time may be able to curb the onset of AD. HupA and J147 are experimental treatments for AD that may function to lower amyloid levels and improve cognitive performance by reducing iron levels. In the human brain, it may be necessary to prophylactically treat with these types of compounds prior to amyloid formation to have an effect on brain health. To do so, the long lasting effects of such therapies and potential gender differences must be investigated.

The discovery that increased iron levels elevate APP expression along the amyloidogenic secretase pathway to efflux cellular iron reveals that iron management is central to the amyloid
hypothesis. An understanding of the iron regulatory systems within neural cells and its link to amyloid production will help in the generation of therapy strategies aimed to curb iron dyshomeostasis and amyloid formation as synergistic contributors to the AD process.

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Chapter 2

Dietary lipophilic iron accelerates regional brain iron load in C57BL6 mice

2.1 Abstract

Impaired brain iron homeostatic mechanisms, independent of pathological hallmarks, are harmful to the brain because excess free iron can cause DNA, protein, and lipid damage via oxidative stress. The goal of this study was to evaluate the longitudinal effect of chronic iron overload and deficiency in the vertebrate brain. Ten-week old C57BL6 male mice were randomly assigned to one of four unique dietary regiments for one year: iron deficient, normal iron, and two different concentrations of lipophilic iron diet containing 3,5,5-trimethylhexanoyl ferrocene (TMHF). Longitudinal MRI parametrics were used to assess the location and extent of ferric iron distribution. Tissue collected at twelve months was used to directly measure iron load, protein alterations, and histological metrics. While the iron deficient diet did not alter brain iron stores, 0.11% TMHF and early dietary exposure with 0.5% TMHF elevated brain iron by roughly 40% and 100%, respectively. R₂ rate increased more in the TMHF groups within iron rich brain regions. Increased brain iron concentration was linearly correlated with an increase in L-ferritin protein expression, and TMHF was found to increase L-ferritin within the olfactory bulb, neocortex, pallidum, thalamus, corpus callosum, CA3 regions of the hippocampus, and substantia nigra. Moreover, gliosis and oxidative stress were detected in the TMHF groups in the regions associated with iron load. Spatial memory impairment was evident in the iron-loaded mice. This work illustrates that lipophilic iron elevates brain iron in a regionally specific fashion and positions dietary TMHF administration as a model for brain iron overloading.
2.2 Introduction

Non-heme iron plays a crucial role in brain metabolic function, serving as a cofactor for the enzymes necessary for biosynthesis of ATP, L-DOPA, cholesterol, and lipids\textsuperscript{254–256}. Iron is important for the growth and development of the brain. However, if not sequestered in a regulated fashion iron can lead to neural dysfunction. As a transition metal, the level and regulation of iron is crucial considering that cycling between ferric (Fe\textsuperscript{3+}) and ferrous (Fe\textsuperscript{2+}) oxidative states produce oxygen radicals that damage lipids, proteins, and unfolded DNA\textsuperscript{257–259}. Elevated brain iron is unique to hominids. Compared to lesser species, the human brain has two-to four-fold more iron by weight than murine brain tissue and naturally increases during brain maturation and aging\textsuperscript{260,261}. In comparison, the levels of other transition metals, such as zinc and copper, are similar amongst vertebrates and do not increase with age\textsuperscript{262}. Increased demand for brain iron is believed to be related to the enlargement of the human brain\textsuperscript{263,264}, as it is necessary for the production and maintenance of the myelin sheath required to conduct electrical signals along association fibers throughout an enlarged cerebrum\textsuperscript{265}. While iron is required in this capacity as part of human brain development, iron dyshomeostasis can occur concomitant to the aging process\textsuperscript{266}. Inducing iron overload and brain iron loading to study this process is difficult because the body and brain tightly regulate iron transport across tissue barriers.

Ferritin is post-translationally regulated in the presence of iron and serves as the primary iron storage protein of the body. However, iron accumulation and oxidative stress to brain tissue occurs when the ferritin complex is disrupted\textsuperscript{267}, such as in AD, Parkinson’s disease, and Restless Leg Syndrome\textsuperscript{104,268,269}. There is a gradual increase in regional human brain iron and ferritin levels plateau during middle-age with some heterogeneity in final concentration\textsuperscript{270,271}. 
Studies examining human brain iron load have linked high human brain iron to impaired recall\textsuperscript{272,273}. Similarly, elevated iron impacts the brain in animal models.

Neonatal mice gavaged 40x the iron content of mouse breast milk within the first two weeks of life showed reduced striatal dopamine and tyrosine hydroxylase positive cells a year later\textsuperscript{274}. Increased brain iron and ferritin levels in hemochromatosis-mutant mice are correlated with increased gliosis, oxidative stress, and memory impairment in older age\textsuperscript{275,276}. Thus, elevating brain iron in earliest days of life set the acceleration of neurophysiological features that normally occur much later.

Many animal models of iron overload during adulthood exist\textsuperscript{277}, but most changes are found peripherally in the heart or liver. Iron salts or sugars cannot freely traverse the gut-blood and blood brain barriers. Administering high doses of iron salts during adulthood has been shown to impair recognition memory\textsuperscript{278,279}. However, most peripheral iron overloading studies do not report brain iron or iron protein changes after administration. We hypothesized that feeding lipid soluble 3,5,5-trimethylhexanoyl ferrocene (TMHF) in adult male mice to traverse the gut and blood brain barriers would better facilitate brain iron overload. TMHF has been utilized in previous studies, but the age of administration, dose and length of dose are mixed. For example, 0.1% TMHF was fed to four-week-old C57BL6 mice for eight weeks\textsuperscript{280}, and three month-old Fischer 344 rats were fed 0.5% TMHF for 14 weeks\textsuperscript{281}. Increased iron content was observed in the cerebrum in the mouse study and increases of the endogenous chelator malondialdehyde was observed in the diencephalon in the rat study. We hypothesized that extended use of TMHF better induces and mimics adult-onset brain iron overload.

While the effects of early brain iron overload on brain neurochemistry are observed later in life, iron deficiency during early life presents with immediate neurological dysfunction. Iron
deficient rat pups demonstrate a reduction in axon diameter and dendritic length which corresponds to reduced action potentials within the cortex and hippocampus\textsuperscript{282}. These neural changes help to explain why chronic iron deficiency in young children impairs psychological and psychomotor development\textsuperscript{283}. Four-week-old C57BL6 mice pups fed an iron-deficient diet for eight weeks had the same brain and liver iron concentration as controls\textsuperscript{280}. We hypothesized that iron deficiency for an extended period of time would lower brain iron stores and induce memory impairment similarly observed in neonatal studies.

2.3 Materials and methods

2.3.1 Dietary Treatment and Animal Care:

C57BL6 male mice were weaned at three weeks and kept on standard diet (2018, Teklad Global, Indianapolis, IN, USA) until they were 10 weeks old. Mice were then fed \textit{ad libitum} one of four diets (Teklad Global, Indianapolis, IN, USA) for 12 months: iron deficient (2-5 ppm trace Fe; n=6), iron sufficient normal iron diet (200 ppm Fe, from ferric citrate; n=6), 0.11\% TMHF (200 ppm Fe; n=6), or 0.50\% TMHF (900 ppm Fe; n=6). Mice fed 0.5\% TMHF were switched to the 0.11\% TMHF diet after three months due to concerns of weight loss. They remained in the study to show the effects of highest dietary iron exposure during development (Fig. 2-1). TMHF was synthesized from 3,5,5-trimethylhexanoyl chloride and ferrocene (Sigma, St. Louis, MO, USA) according to the method of Nielsen and Heinrich\textsuperscript{284}.

Mice were housed in The Pennsylvania State University – College of Medicine animal research facility and cared for by veterinary staff under a twelve-hour light/dark cycle in normal housing conditions as outlined by the Institutional Animal Care and Use Committee (IACUC). All procedures were conducted according to NIH and approved IACUC guidelines. Pregnant dams were fed a high protein diet (Teklad Global, Indianapolis, IN, USA). All animals were
Figure 2-1

<table>
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<th>3</th>
<th>10</th>
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<td>0.5% / 0.11% TMHF DIET</td>
<td></td>
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</tbody>
</table>

0 Mo (Baseline) | 3 Mo | 12 Mo
Figure 2-1. Dietary study design during the twelve-month study

There were four dietary cohorts. All animals were weaned at three weeks of age and kept on normal iron diet until 10 weeks old. At that point they were either kept on the normal iron diet or placed on an iron deficient diet, 0.11% TMHF diet, or 0.5% TMHF diet. The 0.5% TMHF was replaced with the 0.11% TMHF diet after three months. The entire dietary study lasted for 12 months.
kept in standard plastic cages with corn bedding, paper nestlet, stainless steel tops, and an automatic water supply. All animals appeared healthy and survived the duration of the 12-month dietary study.

2.3.2 Barnes Maze:

The Barnes maze consists of a circular table with nineteen empty holes and one exit box around the edge. Black and white sheets surrounded the table with different colored shapes applied at table level. A digital camera, three fans, and lights were fixed to the top of the maze enclosure. Each mouse was placed in a starting box centered on the table for fifteen seconds. The box was then raised and fans turned on to induce movement. At each time point, four three-minute spatial acquisitions were obtained each day for four consecutive days. If the mouse did not find the exit box, it was gently guided to it. The fans were turned off for one minute while the mouse rested in the escape box. Primary, second latency, and the number of errors were measured as a marker of spatial learning and memory. Fewer errors and faster completion were interpreted as better memory\(^{285}\). Short- and long-term memory retention probes were acquired on day five and twelve while the target box was removed\(^{286}\). Memory was measured by nose pokes into the target and error holes\(^{287}\). Distance travelled and speed was measured with AnyMaze behavioral tracking software (Wood Dale, IL, USA). Behavioral differences were observed with 95% confidence intervals from the average across all timepoints.

2.3.3 Plasma Sample:

Mice were fasted for 12 hours, subsequent to 100 µl blood collection from the tail vein with heparinized capillary tubes and kept on ice in gel centrifuge tubes. Blood was centrifuged at 10,000g for 15 minutes at 4°C and plasma was aliquoted with 1:100 protease inhibitor cocktail (Sigma, St. Louis, MS, USA). Samples were kept in -80°C freezer until assayed.
2.3.4 MRI:

Mice were induced with 3-4% isoflurane and anesthesia maintained at 1-2% for the duration of the imaging study. All MRIs were acquired using a 7.0T MRI system with a 23 mm birdcage volume RF-coil (MedSpec 70/20, Bruker BioSpin, Ettlingen, Germany). A whole brain anatomical 3D-RARE dataset with TR = 350 ms, TE = 11 ms, and 100 x 100 x 250 μm final resolution was acquired followed by an eight-echo (11-88 ms) MSME spin-echo R2 dataset with a resolution of 100 x 100 x 500 μm. Raw Bruker images for each subject were preprocessed with in-house qMRI software on an IDL 8.1 platform (Exelis Visual Information Solutions, Inc., Boulder, CO, USA). R2 parametric maps were generated using a nonlinear least squares curve fitting model on a pixel-by-pixel basis288,289 to obtain an accurate relaxation curve fit290 and saved in NIfTI-1 image format. The first echo of the R2 dataset was used as an anatomical marker for spatial alignment to the anatomical 3D dataset. Using the SPMmouse v1.1b toolkit291 within SPM8 (Wellcome Trust Centre for Neuroimaging, UK) each 3D dataset was used to generate a study template image which was co-registered to the relaxation maps followed by the spatial realignment, co-registration, and normalization to the study template image292 using the DARTEL toolbox. Statistical maps had a final voxel resolution of 200 μm isotropic and were smoothed by 400 μm Gaussian kernel. Longitudinal image analysis was performed using an analysis of variance followed by paired t-tests and explicitly masked with a binary whole brain template. Within group comparison of longitudinal data was performed with two-sample t-tests. In both statistical tests, group-wise voxel clusters where considered significantly different with p-values of < 0.005 at a 20-voxel extent threshold. These contrast clusters were output onto the structural template brain and displayed with the SPM slice overlay (slover) output command.
MRI voxel based morphometry was measured every three months of diet to determine brain fractions. A whole brain anatomical 3D T₂-weighted dataset with TR = 350 ms, nine echoes (TE=11 ms, 11 ms spacing), and 100x100x250 μm final resolution was acquired. The SPMmouse package was used to segment the 3D image into GM, WM, and CSF to prepare for DARTEL normalization. Segmented GM and WM tissue classes were imported into DARTEL with SPMmouse before being used to generate a group-specific template image and warps for individual subjects. GM and WM tissue classes were normalized with modulation to create images for use with VBM analysis. Statistical images had a final voxel resolution of 0.2 mm isotropic and were smoothed by a 0.4 mm isotropic gaussian kernel. Paired t-test parametric analysis between baseline and 12-month periods were measured in SPM8. A 20-region mouse brain atlas was overlaid onto the normalized brains to obtain regional VBM measures for the caudate putamen.

2.3.5 Tissue Preparation:

After the 12-month feeding study, mice were anesthetized into a deep surgical plane with ketamine/xylazine (100 mg/kg body weight / 10 mg/kg body weight) and cardiac perfusion was performed with Lactated Ringer’s Solution at 3 ml/min. The brain was removed and hemisected to separate the left and right hemispheres. The right hemisphere was placed in 4% paraformaldehyde for 48 hours followed by 70% ethanol before paraffin embedding. The left hemisphere was dissected into cerebellum, cortex, hippocampus, and basal ganglia. Brain segments were wet-weighed along with a small section of the distal portion of the left liver lobe. RIPA buffer with protease inhibitor cocktail (100:1 ratio) was added to each tissue sample and they were homogenized with plastic pestles. An aliquot was separated for ICP-MS and the remaining volume was spun at 5,000 g for five minutes. Lysate fractions were collected and
protein concentration was measured with a BSA assay (23225, ThermoFisher, Waltham, MA, USA). All lysates were stored in a freezer at −80°C.

2.3.6 ICP-MS:

Brain and liver segments were placed in 1.5 ml centrifuge tubes (Eppendorf) and digested in ultrapure nitric acid (225711, Sigma-Aldrich, St. Louis, MO, USA) at 50°C in an incubator overnight to allow complete acid digestion. Digests were diluted to 5% nitric acid with Millipore water in metal-free positive pressure laboratory. The iron concentration in the acidified brain homogenates was measured with a Thermo Scientific X Series quadropole system with collision cell technology in a metal-free facility. Tissue iron concentration was calculated from a curve generated using National Institute of Standards and Technology standards (1577c, NIST, Gaithersburg, MD, USA). Rhodium was used as the internal reference and method standards (NIST 1640) were used to validate measures. Brain iron was calculated by wet-weight mass fraction.

2.3.7 Enzyme-linked immunosorbent assay (Elisa):

L-Ferritin (ab157713, Abcam, Cambridge, MA, USA) values in brain region lysates were calculated via colorimetric Elisa assay with standard curve normalized to protein concentration. Plasma L-Ferritin (ab157713, Abcam, Cambridge, MA, USA) and C-Reactive Protein (CRP) (ab157712, Abcam, Cambridge, MA, USA) were calculated by standard curve and dilution factor.

2.3.8 Liver enzyme analysis:

Liver homogenate was assayed for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) to determine liver function (Cobas Mira Plus Chemistry Analyzer, Roche Diagnostics Systems Branchburg, NJ, USA).
2.3.9 Blot Analysis:

Lysates were diluted in 1x PBS to a normalized total protein concentration of 5 µg.

Nitrocellulose and filter paper was rinsed in 1xTBST before being placed in a slot blot matrix (Whatman Minifold I, GE Healthcare, Chicago, IL, USA). Wells were rinsed with 1ml of TBST and loaded in duplicate with 5 ug/50 µl of sample per well followed by rinsing with 100 µl TBST under suction to adhere protein to the nitrocellulose. Membranes were blocked in 5% milk for one hour at room temperature. Primary antibodies, diluted in 3% BSA, were added to each membrane and rocked at 4°C overnight: GFAP (1/2000; Rabbit; Dako Z0334), IBA1 (1/500; Rabbit; Wako 016-20001) , HO-1 (1/1000; Rabbit; Enzo ADI-SPA-894-D, and TNFα (1/200; Mouse; Santa Cruz 52746). Blots were washed 3 times for 5 minutes in 1x TBST. Anti-rabbit (1:5000; ECL IgG1 horseradish) and anti-mouse (1/5000; ECL IgG1 horseradish) secondary antibodies were diluted in 1x PBS with 5% milk and rocked at room temperature for one hour. Blots were washed three times for five minutes in 1x TBST before exposing with Western Lightning PLUS ECL followed by imaging with an Amershamb Imager 600 (GE Healthcare Life Sciences). Blots were then rinsed three times in 1x TBST and stripped with Restore Western Blot Stripping Buffer (Thermo Scientific). Blots were rinsed 3 times in 1x TBST and blocked in 5% milk for one hour. Primary staining for β-actin (1/3000; Mouse; Sigma A5441) was repeated in the same manner, and the secondary staining was repeated in the same manner. Densitometry measures were collected with ImageStudioLite software and all antigens of interest were normalized to the β-actin signal.

2.3.10 Histology:

Immunohistology was performed in the same way as described previously. In brief, 5 um sections were cut from paraffin blocks. Sections were plated on clean glass slides and placed
in 50°C incubator for 15 minutes to better adhere tissue to slides. Tissue was rehydrated with in stepwise xylene, ethanol, and water rinses. Slides were placed in boiling hot 10mM citrate buffer (pH 6.0) for 15 minutes and allowed to come to room temperature. Slides were then rinsed in dH2O and blocked in methanol containing 3% H2O2 for 20 minutes. Tissue was then rinsed in pH 7.4 PBS three times and blocked in 2% milk in PBS for one hour. Sections were then stained overnight at 4°C in a humidity chamber with a cocktail of 1% milk, 0.1% Triton-X and one of five different primary antibodies: GFAP (1/1000; Rabbit; Dako Z0334), IBA1 (1/1000; Rabbit; Wako 016-20001), HO-1 (1/500; Rabbit; Enzo ADI-SPA-894-D, TNFα (1/500; Mouse; Santa Cruz 52746), and L-Ferritin (1/1000; Rabbit; Abcam 69090). The next morning slides were rinsed in 1% milk and three PBS washes. Tissue was incubated with 1:200 Vectastain Elite anti-rabbit or anti-mouse HRP conjugated secondary antibody in 1% milk and 0.1% Triton-X for one hour at room temperature. Tissue was then rinsed in PBS three times and counterstained with Vectastain ABC solution for one hour. Tissue was rinsed three times and HRP brown reaction was performed with momentary addition of Vector DAB substrate kit solution. Tissue was rinsed with dH2O and dehydrated with stepwise ethanol and xylene rinses. Slides were coverslipped with non-aqueous mounting media and no. 2 glass coverslides.

Modified Perl’s staining was adapted from methods previously described on 5 um brain sections. Briefly, tissue was deparaffinized with two three-minute SafeClear (xylene substitute) rinses. Iron retrieval was performed with a cocktail of 1% potassium ferrocyanide tri-hydrate (Fisher Scientific P236)/5% polyvinylpyrrolidone (Fisher Bioreagents BP431)/0.05N HCL for 60 minutes. Tissue was quickly rinsed twice with dH2O and placed in methanol containing 0.3% H2O2 for 75 minutes. Tissue was quickly rinsed in PBS twice and stained with cocktail of 0.025% 3,3’-Diaminobenzidine (Sigma-Aldrich D5637)/0.12% H2O2/0.01M pH 7.4
Tris buffer for 10 minutes. Tissue was dehydrated and coverslipped in the same manner described above.

All slides were white balanced to standardize background staining and imaged with Aperio AT2 Leica slide scanner with 40x objective. Virtual slides were randomized by dietary group and a blinded viewer selected a representative brain section by stain and group. Section images were viewed and collected using Aperio ImageScope software.

2.3.11 Statistics:

Statistics for MRI relaxation measures were generated in SPM8 and clusters were set to a \( p \)-value \( \leq 0.005 \) with a 20 voxel extent threshold. Means, standards errors, and T-tests for brain iron and ferritin were derived from an ANOVA with Tukey, Fisher (LSD), and Dunnett’s T3 tests in SPSS 24. Cohen’s-d effect size values for each comparison were calculated from the mean and standard deviation. Outliers were excluded if they exceed 1.5 times the interquartile range above the third quartile or below the first quartile. All graphs and figures were made with DataGraph 4.2 and Adobe Illustrator CS2.

2.4 Results

2.4.1 Animal health was not affected by altered diets:

All animals survived the twelve-month dietary study and did not present with lethargy or anxiety. In a few cases iron-deficient animals presented with pale looking feet near the end of the study and were monitored.

2.4.2 MRI Parametric analysis illustrates regional relaxation changes:

Longitudinal comparison of \( R_2 \) between baseline to three and twelve months are displayed in Figures 2-2a and 2-2b. At three months, \( R_2 \) measures within the iron deficient group presented with increased relaxation rate in the granular layer of the olfactory bulb, right primary
Figure 2-2. Longitudinal relaxation (R2) changes during twelve months of altered iron dietary regimen: Iron deficient, normal iron, 0.11% TMHF and 0.5%/0.11% TMHF

A) Comparison of three months to baseline. B) Comparison of twelve months to baseline.  I) T-score bars illustrate voxels that are significantly different at p-value <0.005: Blue illustrates when baseline measures have greater R2 measures and red illustrates when three or twelve month measures have greater values than longitudinal baseline measures. TMHF appeared to show exacerbated R2 increases in the olfactory bulb, olfactory tubercle, nucleus accumbens, substantia innominate, caudate, globus pallidus and substantia nigra. Normal and iron deficient diets appeared to have bilateral decreases of R2 on distal arms of the corpus callosum.
motor cortex, fimbria, posterior hypothalamic area, right subiculum, and right cerebellar peduncle. The iron-deficient group exhibited decreased $R_2$ in the right external capsule and optic tract. The normal iron group demonstrated a slight $R_2$ increase in the granular layer of the olfactory bulb and nucleus accumbens as well as relaxation rate decreases throughout the external capsule, sensory cortex, primary visual cortex, striatum, medial globus pallidus, hippocampus, cerebellum, and pons. At three months, animals fed the 0.11% TMHF diet had no relaxation decreases, while significant positive differences were present in the anterior olfactory nucleus, nucleus accumbens, caudate putamen, substantia innominata, globus pallidus, cingulate cortex, piriform cortex, lateral dorsal thalamic nucleus, internal and external capsule, midbrain, primary visual cortex, cerebellum, and pons. Animals in the 0.5%/0.11% TMHF group had increased relaxation in the orbital cortex and corpus callosum and negative clusters in the olfactory nerve, cingulate cortex, sensory cortex, and primary visual cortex.

At twelve months, the iron-deficient group had an $R_2$ increase in the granular and glomerular layers of the olfactory, olfactory tubercle, substantia innominate, fundus of striatum, caudate putamen, anterior thalamic nuclei, hypothalamic nuclei, fimbria, hippocampus, primary visual cortex, right cerebellar peduncle, cerebellum, and pons. Significant relaxation decreases were also present within the distal portions of the corpus callosum, the frontal cortex, and the external capsule. The normal iron group presented with significant $R_2$ increases in the granular and glomerular layers of the olfactory bulb, the olfactory tubercle, bilaterally in the substantia innominate, caudate putamen, anterior thalamic pontine central gray, and pons. Bilateral relaxation reduction was observed in the distal portions of the corpus callosum, the same region observed in the iron deficient group. The 0.11% TMHF group did not have any relaxation reduction at twelve months. Significant $R_2$ increases were present in the granular and glomerular
layers of the olfactory bulb, anterior olfactory nucleus, anterior forceps of corpus callosum, globus pallidus, in the nucleus basalis of Meynert (NBM) of the substantia innominata, dorsal lateral thalamic nucleus, cingulate, substantia nigra, sagittal sinus, primary visual cortex, medial geniculate complex, central pontine gray, and cerebellum. The 0.5%/0.11% TMHF group had the largest relaxation increase within the olfactory bulb. Other significant relaxation increases were present in the anterior olfactory nucleus, anterior forceps of corpus callosum, globus pallidus, NBM, dorsal lateral thalamic nucleus, cingulate, substantia nigra, sagittal sinus, and posterior forceps of the corpus callosum. Significant R\textsuperscript{2} reduction was present in the lateral portion of the olfactory nerve, bilateral clusters in the lateral septal nucleus, fourth ventricle, and the central pontine gray.

2.4.3 MRI VBM altered by iron diet:

Parametric analysis of deficient, and normal iron diets illustrates significant decreases in WM volume over time. These changes are observable in several areas of the brain but are most notable within the normal iron group located around the cerebral peduncles, cortical regions, and corpus callosum (Fig. 2-3A). Iron deficient animals also demonstrate a decrease in cortical WM volume. An apparent increase in GM volume is observed in the normal iron animals. Animals on both ferrocene diets appear to have no significant change over time for both GM and WM fractions. MarsBaR analysis reveals that there are broad trends that are similar among deficient, and normal iron diets (Fig. 2-3B and 2-3D). These groups significantly change overtime; WM decreases and grey matter increases. Both ferrocene diets appear to have no volume change for white and gray matter overtime and are significant from the normal iron group at 12 months. 95% Confidence interval trends per dietary group are illustrated in Figure 2-3C and 2-3E.
Figure 2-3

A

<table>
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<tr>
<th>Deficient</th>
<th>Normal</th>
<th>0.1% TMHF</th>
<th>0.1/0.5% TMHF</th>
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</table>

Gray Matter Fraction

White Matter Fraction

B

Caudateputamen

VBM Grey Matter (A.U.)

Iron Diets

Deficient Iron | Sufficient Iron | Normal Iron | 0.1% TMHF | 0.1/0.5% TMHF

D

Caudateputamen

VBM White Matter (A.U.)

Iron Diets

Deficient Iron | Sufficient Iron | Normal Iron | 0.1% TMHF | 0.1/0.5% TMHF

C

Caudateputamen - VBM Grey Matter Fraction

VBM Grey Matter (A.U.)

95% Confidence Intervals

- Deficient iron diet
- Sufficient iron diet
- Normal iron diet
- 0.1% TMHF diet
- 0.1/0.5% TMHF diet

E

Caudateputamen - VBM White Matter Fraction

VBM White Matter (A.U.)

95% Confidence Intervals

- Deficient iron diet
- Sufficient iron diet
- Normal iron diet
- 0.1% TMHF diet
- 0.1/0.5% TMHF diet
Figure 2-3. Voxel Based Morphometry (VBM) parametric comparison diets and of caudate-putamen WM and gray matter fraction

A) Parametric analysis of VBM grey and WM fractions. Paired t-test between group baseline VBM measures and 12 month VBM measures. There is an apparent decrease in WM in the deficient, and normal iron groups. This decrease is shown in the cerebral peduncles, fimbria, corpus callosum, caudate, and areas of the cortex. Conversely there is an apparent increase in gray matter in the normal iron group. For example, there is a significant difference around the medial lemniscus in the normal iron group. These changes are not observed in the TMHF groups. Statistical images had a final voxel resolution of 0.2mm isotropic and were smoothed by a 0.4 mm isotropic Gaussian kernel. Parametric analysis of VBM was performed in SPM8. P value ≤ 0.001. Caudate-putamen mean VBM values for grey B) and WM fractions C) between different iron diets. Intervals are demarcated by 3-month intervals. 95% CIs between groups over 12 months in grey D) and white E) matter fractions. This indicates that there are broad grey and WM changes in a normal mouse brain that are altered when fed a TMHF diet. These trends are similar in most of the other 20 brain regions observed.
2.4.4 Brain iron and L-Ferritin increase with TMHF diets:

Chronic 0.5%/0.11% and 0.11% TMHF diet administration for twelve months elevated brain iron and L-ferritin stores by as much as 100% and 40%, respectively. Table 2-1 illustrates mean protein concentration across different dietary groups and brain regions. All t-test and effect size statistics are in comparison to normal iron diet. The 0.11% and 0.5%/0.11% TMHF diets resulted in a significant increase in cerebellar L-ferritin relative to normal iron diet. The 0.5%/0.11% TMHF diet significantly increased L-ferritin in the caudate putamen and cortex. The 0.5%/0.11% TMHF also had a significant increase in cerebellar iron concentration. Large effect sizes (>1.0) were observed for all TMHF diets in the cerebellum, caudate putamen and cortex. The 0.5%/0.11% TMHF diet had an increased effect relative to the 0.11%TMHF diet in the cerebellum and caudate putamen. A non-significant trend was observed for hippocampal iron and L-ferritin.

Figure 2-4 displays correlations of concatenated brain L-ferritin to iron for four different brain regions: cerebellum, caudate putamen, cortex, and hippocampus. Correlation coefficients were 0.26, 0.26, 0.87 and 0.58 for iron deficient, normal iron, 0.11% TMHF and 0.5%/0.11% TMHF diets, respectively. The 95% confidence intervals were used to illustrate group differences. Confidence intervals overlapped for iron-deficient and normal iron diets. The 95% confidence intervals for the 0.11% TMHF dietary group were higher than normal and iron deficient groups, and the 95% confidence interval for the 0.5%/0.11% TMHF group was significantly greater than the 0.11% TMHF group’s.

2.4.5 Liver iron and plasma proteins increase with TMHF diets:

ICP-MS measured liver iron concentration for the four groups was 41 ppm (iron deficient), 173 ppm (normal), 4007 ppm (0.11% TMHF), and 4799 ppm (0.5%/0.11% TMHF)
## Table 2-1

<table>
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<td>4.1E-04* 4.1E-05</td>
<td>3.1E-04 4.0E-05*</td>
<td>3.1E-04 3.3E-04* 4.0E-04*</td>
</tr>
<tr>
<td>Iron Deficient</td>
<td>7.8E-04*** 7.8E-05</td>
<td>5.5E-04 5.5E-05*</td>
<td>2.6E-04 2.6E-04 4.0E-04*</td>
</tr>
<tr>
<td><strong>Iron</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal Iron</td>
<td>16.8</td>
<td>1.6</td>
<td>19.7</td>
</tr>
<tr>
<td>Iron Deficient</td>
<td>18.1</td>
<td>1.7</td>
<td>18.8</td>
</tr>
<tr>
<td>0.11% TMHF</td>
<td>21.3</td>
<td>1.7</td>
<td>26.8* 17.9*</td>
</tr>
<tr>
<td>0.5%/0.11% TMHF</td>
<td>26.6*** 1.6</td>
<td>26.2* 1.6</td>
<td>23.2** 1.7</td>
</tr>
</tbody>
</table>
Table 2-1. Regional brain iron and L-ferritin concentrations after twelve months of dietary regimen

L-ferritin was measured by ELISA and normalized to total lysate protein content. Iron concentration was measured with ICP-MS and normalize by tissue wet weight. A) Means and standard errors for each group. Fisher’s least significant difference (LSD), and effect size was measured by Cohen’s D; Significantly different than normal iron dietary group: * \( p \)-value \( \leq 0.05 \), ** \( p \)-value \( \leq 0.01 \), *** \( p \)-value \( \leq 0.001 \); Significantly different than 0.11% TMHF group \( \dagger \) \( p \)-value \( \leq 0.05 \), \( \dagger \dagger \) \( p \)-value \( \leq 0.01 \), \( \dagger \dagger \dagger \) \( p \)-value \( \leq 0.001 \); Effect size greater than normal iron dietary group \( \Delta \) Cohen’s D \( \leq 1.0 \), \( \Delta \Delta \) Cohen’s D \( \leq 2.0 \), \( \Delta \Delta \Delta \) Cohen’s D \( \leq 3.0 \).
Figure 2-4

Brain Iron to L-Ferritin by Diet

- Iron Deficient: $R^2=0.4177$
- Normal Iron: $R^2=0.514$
- 0.11% TMHF: $R^2=0.8717$
- 0.5%/0.11% TMHF: $R^2=0.7344$

L-Ferritin (ng)/Total Protein (ng) vs. Fe (ug)/ tissue wet weight (g)
Figure 2-4. Regional brain iron and L-ferritin concentrations after twelve months of dietary regimen

Correlation of L-ferritin to regional brain iron was concatenated and displayed by 95% CI by dietary group excluding outliers. Iron-deficient and normal iron diet plots overlapped while the slope of ferritin to iron increased with each TMHF diet.
An ANOVA with post-hoc test demonstrated significance between the deficient to normal iron and normal to TMHF diets. There was no significant difference between the two TMHF diets. The mean plasma L-ferritin concentration for all groups at baseline (0 months) was 1153 ng/mL with no significant difference between groups. The control diet iron plasma ferritin, 2202 ng/ml, was significantly higher at three months. Plasma ferritin in the control iron diet group appeared to plateau at three months and was not different from the groups mean plasma ferritin at twelve months, with a mean value of 2235 ng/ml (Fig 2-5B). Iron-deficient diet plasma ferritin was slightly elevated at three months, 1529 ng/ml, but was not significantly different. However, the plasma ferritin in the deficient iron group was significantly lower than all other groups at twelve months, 974 ng/ml. The 0.11% TMHF group’s three-month plasma ferritin was significantly elevated with a concentration of 81,171 ng/ml, while the twelve-month value for this group was significantly elevated to 95,663 ng/ml. The 0.5%/0.11% TMHF group’s plasma ferritin was significantly different and was six-fold higher than the 0.11% TMHF group at three-months. However, after having been placed on the 0.11% diet at the three-month time point, the 0.5%/0.11% TMHF group’s plasma ferritin was similar to the 0.11% group’s ferritin at twelve months with a value of 115,385 ng/ml.

At twelve months, there was no difference between dietary groups for plasma measurement of liver enzymes asparate and alanine aminotransaminase (Fig. 2-5C). The C-reactive protein was not increased in the two TMHF dietary groups’ plasma (Fig. 2-5D). The TMHF measures were slightly lower at twelve months compared to the normal and deficient iron groups. At twelve months, the iron-deficient group had significantly higher plasma C-reactive protein than the normal iron group.
Figure 2-5. Liver iron, plasma L-ferritin, aspartate (AST)/alanine (ALT) transaminase enzymes, and acute phase c-reactive protein (CRP) analysis

A) Deficiency lowers liver iron three-fold while TMHF elevates liver iron 20-fold over twelve months. B) Circulating ferritin concentration appears to be dose dependent on iron stores. At twelve months plasma ferritin is two-fold lower in the deficient group and 50-fold higher in the TMHF groups. C) AST/ALT liver enzymes did not indicate cirrhosis or liver damage in any dietary group, and D) C-reactive protein is not elevated in the TMHF groups meaning that liver function is preserved with chronic TMHF diet and circulating ferritin is independent from peripheral acute phase reactions. Significance was determined by Tukey post hoc test or Dunnett’s T3 tests dependent on the group variance homogeneity. * $p$-value $\leq 0.05$; ** $p$-value $\leq 0.01$; *** $p$-value $\leq 0.001$. 
2.4.6 **Blot Analysis shows increased gliosis and oxidative stress with TMHF:**

Glial, oxidative stress, and inflammation markers were regionally measured to determine the effect of dietary iron overload and the regional relationship to brain MRI relaxation parameters. GFAP and HO-1 significantly trended highest in the TMHF groups for all regions observed (Fig. 2-6). Normalized HO-1 and GFAP measurement in the basal ganglia were 67% and 55% greater, respectively, in the TMHF group than in the normal iron group. The normal iron group had significantly higher GFAP levels than the iron-deficient group in the basal ganglia (151%) and neocortex (29%). IBA1 was significantly greater in the TMHF groups’ basal ganglia (180%) and neocortex (109%) compared to the normal iron group, while TNFα was significantly higher in the TMHF groups’ basal ganglia (271%).

2.4.7 **Histology validates protein quantification and MRI:**

Figure 2-7 illustrates qualitative assessment of Perl’s stain. Neuronal iron and parenchymal staining within the olfactory bulb granule and glomerular layers appear darker in both TMHF dietary groups compared to deficient and normal iron diets. Similar observations were made in the primary motor cortex, corpus callosum, pallidum, olfactory cortex, posterior thalamus, substantia nigra, interpeduncular nucleus, and the CA3 pyramidal layer of the hippocampus. The choroid plexus within the lateral ventricle also stained strongly for iron in the TMHF dietary groups. Iron staining appeared similar in the hypothalamus and the anterior commissure in all dietary groups.

L-Ferritin staining within the brains of the different dietary groups (Fig. 2-8) reflects the iron quantitative dose-dependent increase illustrated in Table 1 and Figure 2-3. L-ferritin was highly expressed in the olfactory bulb of all dietary groups, but was increased in the TMHF diets. L-ferritin significantly stained apparent neurons and glia throughout the brain, especially within
Figure 2-6

Cerebellum

Basal Ganglia

Neocortex

Hippocampus

Antigen of Interest

Signal/Actin Signal

IBA1, GFAP, TNFα, HO1
Figure 2-6. Protein blot analysis of brain regions where antigen signal was normalized to actin Microglial (IBA1), Astrocyte (GFAP), and oxidative stress (HO-1) markers are elevated in most brain regions with elevated brain iron and ferritin. TNFα (acute phase reaction and inflammation) was elevated in the basal ganglia. These results reflect MRI findings where most observed differences were found in the basal ganglia. * $p$-value $\leq 0.05$; ** $p$-value $\leq 0.01$; *** $p$-value $\leq 0.001$. 
Figure 2-7
Figure 2-7. Representative Perl’s stain of different brain regions for each dietary group

Numbers above represent position of brain area to bregma. Dorsal regions of interest include: granule layer of the olfactory bulb, primary motor cortex, corpus callosum, hippocampal CA3 pyramidal layer, and hippocampal CA1 field. Ventral regions of interest include: glomerular layer of the olfactory bulb, ventral striatum, nucleus basalis of meynert (NBM), posterior thalamic nuclei, and substantia nigra. The iron-deficient and normal iron dietary groups visually stain for similar amounts of iron. Contrast is standardized across all images to the background to allow comparison between groups. Scale bar of magnified regions of interest are 250µm; scale bar below the large whole section view is 1mm.
Figure 2-8
Figure 2-8. L-ferritin stain of the olfactory bulb, substantia innominata, neocortex, corpus callosum, hippocampal regions, and substantia nigra

ROI views match Perl ROIs in Figure 2-6. Qualitative inspection suggests that increased brain iron parallels L-ferritin deposition. Scale bar is 250 µm.
the olfactory bulb, neocortex, substantia innominate, hippocampus, posterior thalamus, and substantia nigra. L-ferritin ‘starburst-like’ deposits, evidence of microglial cytorrhexitis or hemosiderin deposition, were found in the CA1, CA2, and CA3 hippocampal fields in all dietary groups, but was more apparent in the TMHF groups.

IBA1 staining of different dietary group brains revealed TMHF increased number of amoeboid phagocytic microglia within the olfactory bulb and motor cortex (Fig. 2-9). Microglia appeared more numerous and ramified within the pallidum, substantia innominate, CA3 hippocampal field, and posterior thalamus of the TMHF dietary groups’ brains. The iron deficient diet visually appeared to have less IBA1 staining throughout the mouse brain. TMHF increased HO1 staining is predominantly found in highly vascularized regions of the brain, such as the hippocampus and olfactory bulb (Fig. 2-10A). TNFα staining mimicked HO1 staining, but to a lesser degree. There was a subtle difference in TNFα staining by diet (Fig. 2-10B). GFAP staining did not appear different between dietary groups (not shown).

2.4.8 Barnes Maze spatial learning is impaired with TMHF:

Both 95% CI from the TMHF groups overlap across all four training days and are significantly higher than that of the iron deficient and normal iron groups for the first three training days (Fig. 2-11A). Thus, TMHF groups had impaired spatial memory relative to normal and iron-deficient groups. The iron-deficient and normal iron dietary group error trends overlap for all training days. Memory probes (Fig. 2-11B) demonstrated that there is not a significant difference in memory retention between the different dietary groups. Average speed during the probe trials (Fig. 2-11C) was not significantly different for the first nine months of testing. However, the iron-deficient group was significantly slower at the last twelve-month time point.
Figure 2-9. IBA1 stain of the olfactory bulb, substantia innominata, neocortex, corpus callosum, hippocampal regions, and substantia nigra

ROI views match Perl ROIs in Figure 2-6. IBA1 staining illustrates numerous amoeboid-like microglia, in areas of the brain that stain heavily for iron. Qualitative inspection suggests that increased brain iron and L-ferritin correlates with IBA1-positive microglia. Scale bar is 250 µm.
Figure 2-10

**A. Heme oxygenase 1 (HO-1)**

Off. Bulb - Glomerular Layer  | Nucleus Basalis of Meynert  | Corpus Callosum  | CA3 - Pyramidal Layer  | CA1 Field
---|---|---|---|---
DEFICIENT
NORMAL
TMHF

**B. Tumor necrosis factor alpha (TNFα)**

Off. Bulb - Glomerular Layer  | Nucleus Basalis of Meynert  | Corpus Callosum  | CA3 - Pyramidal Layer  | CA1 Field
---|---|---|---|---
DEFICIENT
NORMAL
TMHF

250 μm
Figure 2-10. Representative brain areas that showed HO-1 and TNFα differences by diet

A) HO-1 stained heavily in the olfactory bulb and hippocampus of the mice fed TMHF. B) TNFα matched HO-1 staining in the hippocampus, but was relatively absent from the majority of the brain in all dietary groups. Scale bar is 250 µm.
Figure 2-11

A. Average Training During 12 Month Study -- Primary Error

B. Average Probes During 12 Month Study -- Error Pokes/Target Pokes

C. Average Speed During Probes
Figure 2-11. Barnes escape maze results

Animals were tested every three months of diet and results presented with 95% confidence intervals. A) Average of primary errors per diet group over the twelve-month course of study. TMHF groups appeared to have similar deficits as compared to normal and iron deficient diets. There were no differences between iron deficient and normal iron diets. B) 95% CI of average errors/pokes during the memory task were not different across groups, so spatial memory was not significantly altered by any diet. C) 95% CI of average speed during probe tasks overlapped over the course of the study, so we conclude motor function was not impaired in any of the experimental diets.
2.5 Discussion

Chronic administration of TMHF over a twelve-month period increases brain iron concentration in a regionally selective manner and impairs spatial learning, while adult iron deficiency does not alter brain iron or behavior. The results expand upon previous research where increases in systemic iron storage have been demonstrated with dietary ferrocene administration\textsuperscript{280,281} and provides a new model for regional iron loading in the adult brain.

We evaluated liver iron and circulating L-ferritin to better understand the temporal effect of peripheral iron stores on murine brain iron and found that circulating L-ferritin and liver iron levels are positively associated with dietary iron concentration. Plasma L-ferritin in the iron-deficient diet group persisted within a normal range for the initial months on the deficient diet but after 12-months of deficient iron intake, when liver iron content was also found to be significantly lower, plasma ferritin was reduced. Conversely, brain iron may elevate when peripheral iron levels are high. Circulating plasma L-ferritin at three months was found to be five times higher in the 0.5%/0.11% TMHF group than the 0.11% TMHF group. This difference was no longer present when the 0.5% TMHF diet was replaced with the 0.11% TMHF diet. The early increase in peripheral or acute iron load may explain why the 0.5%/0.11% TMHF group had greater brain iron concentration at 12 months. Further investigation to evaluate TMHF penetrance of the BBB and iron transport regulator, hepcidin, may elucidate the impact of peripheral iron stores on brain iron load. The fast influx and slow efflux theory proposed by Chen et al. may explain why deficient animals had the same brain iron as control diet and why 0.5% TMHF had a lasting effect on total brain iron\textsuperscript{295}.

MRI $R_2$ rate is positively correlated to brain iron concentration in AD\textsuperscript{296} and disease progression in neuroferritinopathy\textsuperscript{297}. $R_2$ parametrics are a reproducible method\textsuperscript{298} used to
measure iron overload in the brain as well as other organ systems where an increase in R₂ rate is indicative of iron loading. Conversely, a decrease in MRI relaxation rate can be the result of many factors including the presence of inflammation, edema, decrease in iron concentration, or loss of proton compartmentalization. There was a consistent R₂ increase at twelve months in the olfactory bulb, ventral pallidum, and ventral striatum within all dietary groups; however, the increase of R₂, L-ferritin, and Perl’s staining was most evident in the TMHF fed mice. Our results suggest that iron-rich areas of the brain sequester and store excess brain iron during extreme iron load even though they apparently have equal access to the lipophilic TMHF compound. Further, the focal ferritin deposition within the olfactory bulb and pallidum in TMHF groups seemed to parallel brain L-ferritin levels and MRI R₂ differences, supporting the idea that increased ferritin polymerization increases R₂. The focal deposition of brain iron within ferritin via TMHF supplementation appears to drive MRI relaxation differences between dietary groups more so than purely increasing total brain iron.

In addition to areas that were prone to accumulate iron, there are areas that seem vulnerable to lose iron. Loss of the cellularity in the genu of the corpus callosum was observed in the iron deficient and normal iron groups. The callosal genu is a late myelinating region with small fiber bundles that are most vulnerable to age and disease modulated demyelination in mice and humans. This finding supports the retrogenesis model within late myelinating regions of the corpus callosum. The TMHF dietary groups did not show these WM hypointensities and had stronger Perl’s staining, suggesting that increased WM iron may have an effect on maintaining axonal integrity.

There was a higher concentration and increased staining intensity of GFAP, IBA1, and HO1 within the neocortex and basal ganglia in the TMHF groups. Astrocytic differences were
difficult to qualitatively compare across groups, whereas IBA1 number appeared to increase in brain areas where iron was most abundantly increased by TMHF diet, such as the primary cortex and olfactory bulb. This may reflect increased microglial activity. TNFα levels did not match increased glial markers, ferritin or iron concentration, and TNFα staining was weak in the brains of all dietary groups. Thus, increased brain iron activated glial cells are present without initiating a significant inflammatory event or acute phase reaction. This observation matches what we found in the periphery.

C-reactive protein did not change in the presence of increased circulating ferritin. The lack of an acute phase reaction in the brain and periphery, without visible alterations of circulating ALT and AST, support the notion that TMHF or deficient iron diets do not lead to acute liver failure$^{307}$. Thus, hepatic encephalopathy can be discounted as a potential confounder of physiological or behavioral differences due to different iron diet. TMHF serves as a molecular vehicle to elevate brain iron and gliosis without the additional problems associated with a systemic inflammatory agent, such as lipopolysaccharide$^{308}$. Therefore, behavioral deficits attributed to elevated brain iron and gliosis better mimic senescence.

Behavioral measures indicate that dietary TMHF impairs spatial learning while iron deficiency does not. Impaired memory with the TMHF groups parallels iron deposition within focal areas of the pallidum and basal ganglia, such as the NBM. The basal ganglia is responsible for non-declarative memory formation, such as habitual learning and is believed to be interconnected with other memory systems, such as the temporal lobe, for declarative memory. Bartzokis et al. demonstrated that Alzheimer’s patients are more likely to have higher iron levels in the basal ganglia than healthy controls$^{309}$. Future studies to investigate the correlation
between behavioral deficit, iron concentration, and pathological features may highlight the temporal relationship between these variables.

The NBM is a posterior cholinergic nuclei of the substantia innominate within the ventral pallidum that has been shown to have significant neuronal loss during neurodegenerative disease and is suggested to be a site of therapy to reduce symptoms of dementia. An increase in iron has been demonstrated in the NBM in AD patients. The nucleus basalis is an interface island of nutrient-rich densely packed cells that may constitute a reserve to facilitate adaptive structural networking of the brain. Lesions to the substantia innominata disrupt ipsilateral blood flow to the frontal cortex and striatal cortex. The data from the TMHF fed mouse suggest this region is susceptible to increased iron loading. We demonstrated that iron deposition, gliosis and inflammation in the NBM are linked to spatial learning deficits that mimic AD.

In conclusion, this work demonstrates that iron deficiency during adulthood does not perturb iron balance in the brain and that iron loading with TMHF is tolerated and leads to increased brain iron and ferritin in the adult mouse. Longitudinal measures showed an increase in brain iron after three months in regions that normally acquire iron with age. Furthermore, brain iron substantially increases during adulthood, even under iron-replete conditions. Our findings indicate that iron is processed differentially by brain region and demonstrate that regions normally high in iron sequester rather than process iron out of the region. The results position dietary TMHF as a model for brain iron overloading observed in neurocognitive disorders.
2.6 Acknowledgements

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Chapter 3
Dietary lipophilic iron regional increases brain and amyloid iron load

3.1 Abstract

AD (AD) is a progressive neurodegenerative disease characterized by Aβ (Aβ) deposition, microgliosis and iron dyshomeostasis. An increasing labile iron pool of the aging brain is believed to increase Aβ formation. Free iron levels can intercalate into aggregating amyloid peptides increasing their oxidative stress potential. Our previous work found that human Aβ plaques stain intensely with iron and activated microglia while transgenic mouse plaques stain with much less intensity. The goal of this work was to observe how increasing and decreasing brain iron influenced Aβ levels, amyloid plaque iron loading, and microgliosis. We fed the lipophilic iron compound 3,5,5-trimethylhexanoyl ferrocene (TMHF) and iron deficient diets to humanized Aβ knock-in mice, NL-F and NL-G-F, for twelve months. TMHF elevated brain iron by 22% and iron deficiency decreased brain iron 21% relative to control diet. Increasing brain iron decreased Aβ40 plaque load, elevating the Aβ42/40 plaque load ratio and senile morphology of amyloid plaques. Increased brain iron was associated with increasing plaque-iron load and microglial iron inclusions. TMHF decreased IBA1+ microglia branch length while increasing microglial roundness. This body of work suggests that increasing mouse brain iron with TMHF potentiates a more human-like AD phenotype with iron integration into Aβ plaques and associated microgliosis.
3.2 Introduction

The human brain is rich in iron as compared to other vertebrate brains\textsuperscript{71,317}. Iron serves as a cofactor for a host of enzymatic processes that facilitate our advanced neuroarchitecture and myelination during development\textsuperscript{264}. Iron deficiency alters normal structure and function of WM\textsuperscript{282} leading to lasting cognitive impairment\textsuperscript{318}. In addition, increased iron deposition is observed in the AD (AD) brain\textsuperscript{319}. The amyloid precursor protein (APP) is upregulated in the presence of free iron because it contains an iron-responsive element (IRE) in its 5'-untranslated protein transcript\textsuperscript{53}. Evidence suggests that one function of APP is to facilitate export iron across the neuronal plasma membrane\textsuperscript{124}. Cleaved APP protein fragments produce Aβ (Aβ) plaques that accumulate iron deposits. These plaques are believed to focally increase iron’s oxidative stress potential\textsuperscript{320} by serving as a reductant\textsuperscript{62}.

We previously found that iron in the AD brain is associated with microglial dystrophy\textsuperscript{9}, and recent work has shown that the iron positive microglia in the hippocampus differentiate healthy and AD brains\textsuperscript{268}. Increased iron in microglia results in phagocytosis\textsuperscript{321} and polarization to a detrimental M1 inflammatory phenotype\textsuperscript{322}. Human Aβ plaques are morphologically distinct from previous transgenic mouse Aβ plaques; human Aβ plaques contain greater levels of iron and phagocytic microglia than transgenic mouse Aβ plaques\textsuperscript{7,293}. We suggest that increased human brain iron levels observed in AD may initiate the formation of iron-rich Aβ plaques, leading to further microgliosis and toxicity.

The goal of this study was to alter the mouse brain iron to better mimic human brain conditions associated with AD etiology and pathology. We increased and reduced brain iron levels in the transgenic mouse brain to better understand how regional iron levels impact
amyloidosis and microgliosis. We fed a lipophilic 3,5,5-trimethylhexanoyl ferrocene (TMHF) and iron-deficient diet to the newly developed transgenic AD mouse with familial APP knock-in mutations. This animal model is hypothesized to respond to the effect of iron loading and iron deficiency because the APP transgene is expressed on the endogenous mouse APP promoter with its native IRE sequence. Our previous work in a C57BL6 mouse demonstrated that TMHF elevates brain iron in a regional manner and impairs spatial memory. We hypothesize that artificially increasing brain iron will promote Aβ plaque iron burden, alter plaque morphology, modify amyloidogenesis, and amplify subsequent microgliosis.

3.3 Materials and Methods

3.3.1 Dietary Treatment and Animal Care:

We utilized two different APP knock-in mutant mice: APP\textsuperscript{NL-F} (NL-F) that slowly generate Aβ and APP\textsuperscript{NL-G-F} (NL-G-F) that rapidly generate Aβ. We weaned the mice at three weeks and randomly separated them equally by sex onto one of three different diets ad libitum for 2, 6, and 12 months (Fig 3-1): iron-deficient (2-5 ppm trace Fe; n=6/timepoint/strain/sex/diet), normal iron diet (200 ppm Fe, from ferric citrate; n=6/timepoint/strain/sex/diet), and 0.11% TMHF (200 ppm Fe; n=(6/timepoint(3)/strain(2)/sex(2)/diet(2))=216. TMHF was synthesized from 3,5,5-trimethylhexanoyl chloride and ferrocene (Sigma, St. Louis, MO, USA) as previously described.

Mice were housed at The Pennsylvania State University – College of Medicine animal research facility, and cared for by veterinary staff under a twelve-hour light/dark cycle in normal housing conditions as outlined by the Institutional Animal Care and Use Committee (IACUC). We conducted all procedures according to NIH and approved IACUC guidelines. Pregnant dams
Figure 3-1. Study design during the twelve-month dietary iron experiment with three dietary cohorts

All animals were weaned at three weeks of age then were placed on the deficient, normal, or TMHF iron diet for 12 months. Cohort tissue was collected at 2 month, 6 month, and 12 month timepoints.
were fed a high protein diet (Teklad Global, Indianapolis, IN, USA). Animals were kept in standard plastic cages with corn bedding, paper nestlet, stainless steel tops, and an automatic water supply. We observed no obvious health problems as a result of the dietary study.

3.3.2 HPLC of serum and brain lysates:

Non-study NL-G-F mice (n=2♀/2♂) were utilized from the same housing facility to identify the presence and concentration of TMHF in the serum and brain. Six-month-old animals were placed on 0.11% TMHF for two weeks. Afterwards, animals were fasted for 10 hours and retro-orbital blood from one male and one female were collected in an alternated fashion every two-hours beginning at baseline with a clean glass pipette. Mice were anesthetized with 3-4% isoflurane and approximately 50 µl of blood was collected at each time. Blood was placed in gel centrifuge tubes and spun at 10,000 g for 15 minutes at 4°C. Serum was kept at -80°C prior to HPLC analysis. Animals were placed back on 0.11% TMHF diet for four hours and were observed eating the pellets. Animals were then fasted overnight for approximately 10 hours.

Mice were anesthetized into a deep surgical plane with ketamine/xylazine (100 mg/kg body weight / 10 mg/kg body weight) and cardiac perfusion was performed with Lactated Ringer’s Solution at 3 ml/min. The mouse brain was segmented into four sections: olfactory bulb, cerebellum with hindbrain, cortex, and subcortical tissue. Brain segments was blotted dry, weighed, diluted with 100 RIPA buffer:1 Protease inhibitor cocktail / 1 mg tissue, pestle homogenized, placed on ice, and sonicated. Homogenates were then centrifuged at 18,000 g for 20 minutes at 4°C and kept lysates at -80°C prior to HPLC analysis.

Serum and brain lysates were extracted with acetonitrile/H₂O/formic acid (90/10/0.1 ratio). Samples were vortexed and centrifuged at 4 °C at 8,765 g for 10 min. The supernatant
was dried down by Speed Vac and was then reconstituted by acetonitrile/H₂O/formic acid (50/50/0.1 ratio) before loading onto the UPLC/MS/MS system.

Samples were analyzed with an ABSciex 4000 Q Trap mass spectrometer coupled with a Waters Acquity UPLC separation system. A 1.7 μm Acquity UPLC BEH C18 analytical column (2.1 x 100 mm, Waters, Ireland) was used to separate TMHF from any other interference. The gradient elution was conducted using a flow rate of 0.3 mL/min with the following conditions: initial 5% mobile phase B (acetonitrile), 95% solvent A (0.1 % formic acid in water), a linear gradient to 100% mobile phase B for one minute, and 100% mobile phase B was used for three minutes to flush the column. The autosampler was kept at 4 °C, and the column temperature was maintained at 30°C. For quantification, the multiple reaction monitoring mode (MRM) was used to analyze TMHF with the transitions of \( m/z \) 327 > 141. Unknown sample concentration was determined by comparing peak area to known standard curve concentrations that were made from our original synthesized TMHF. The unknown sample concentrations were calculated by volume.

3.3.3 Blood Analysis:

Ten μl of tail whole blood was collected with EDTA-K2 plastic pipettes (Boule; 1070039; Sweden) and immediately run on a Mindray BC-2800 Auto Hematology Veterinary Blood Analyzer along with 3PD Hematology Control Standards (CDS NextGeneration™; 502-004; USA). White and red blood cell information was utilized to evaluate potential infection and effect of iron overload on blood measures. Mouse strain and sex were concatenated to observe the effect of diet on blood parameters. Table 1 depicts mean values, standard error, and 95% confidence intervals (CI) for all time points. P-values were calculated to compare within diet
group differences over time and across diet at each timepoint. A significant difference to normal and 0.11%TMHF groups was determined with Fisher’s least significant difference (LSD).

3.3.4 Tissue Preparation:

After the 2, 6, or 12-month dietary regiment, mice were anesthetized into a deep surgical plane with ketamine/xylazine (100 mg/kg body weight / 10 mg/kg body weight) and cardiac perfusion was performed with Lactated Ringer’s Solution at 3 ml/min. The brain and liver were extracted, and the brain was hemisected to separate the left and right hemispheres. Our previous work demonstrates that iron loading with TMHF is distributed bilaterally. The left hemisphere and liver was flash frozen in 2-methylbutane and kept at −80°C until homogenization. The right hemisphere was placed in 4% paraformaldehyde for 48 hours followed by 70% ethanol before paraffin embedding. Half the animals were randomly selected and sliced for histological analysis (n=3/timepoint/strain/sex/diet). Tissue was sagittaly cut at 5µm and mounted on slides for staining and LA-ICP-MS.

Left hemisphere tissue was thawed and the olfactory bulb and cerebellum were removed with a perpendicular cut to the rostral isocortex and inferior colliculus with a stainless-steel scalpel. The liver was thawed, and the distal section of the right lobe was removed and weighed for ICP-MS analysis. The left brain section was then weighed and homogenized in RIPA: Protease inhibitor cocktail (100:1 ratio) with plastic pestles. An aliquot was separated for ICP-MS and the remaining volume was sonicated and spun at 18,000 g for 20 minutes. Lysate fractions were collected and protein concentration was measured with a BSA assay (23225, ThermoFisher, Waltham, MA, USA). All lysates were stored in a freezer at −80°C.
3.3.5 ICP-MS:

Brain homogenate aliquots and liver segments were placed in 1.5 ml centrifuge tubes (Eppendorf) and digested in ultrapure nitric acid (225711, Sigma-Aldrich, St. Louis, MO, USA) at 50°C in an incubator overnight to allow complete acid digestion. Digests were diluted to 5% nitric acid with Millipore water in metal-free positive pressure laboratory. The iron concentration in the acidified brain homogenates was measured with a Thermo Scientific X Series quadrupole system with collision cell technology in a metal-free facility. Tissue iron concentration was calculated from a curve generated using National Institute of Standards and Technology standards (1577c, NIST, Gaithersburg, MD, USA). Rhodium was used as the internal reference and method standards (NIST 1640) were used to validate measures. Brain iron was calculated by wet-weight mass fraction. Group means were calculated and the effect of diet, sex and strain on tissue iron was evaluated with SPSS 22 (IBM, New York, USA). The effect was scored by Wilk’s Lambda partial eta squared values and significant differences between groups were acknowledged with an alpha ≤ 0.05.

3.3.6 LA-ICP-MS:

For qualitative iron analysis, one animal from each group was selected at the six and twelve-month time points (n=1/time point/strain/sex/diet). Slides were deparaffinized in SafeClear as previously described to reduce loss of iron from xylene/ethanol rinses. Slides were kept in a dry and metal-free container until ablated. Tissue was ablated in a similar manner as performed previously. Briefly, sections were imaged at 50µm spatial resolution (total area = 2.5 mm²/pixel) and were ablated with a 193 nm New Wave excimer ArF source laser connected to a Thermo X-Series II quadrupole ICP-MS instrument. Energy fluence was set at 0.35 J/cm² and laser power was 3% combined with a high repetition rate of 20 Hz to ensure
proper ablation of tissue. Ablation lines were set parallel with a 7µm space, and the ablation x and y coordinates were set so that 43µm x 50µm spot would fit evenly for image reconstruction. We ablated the tissue at 250 µm/s and measured $^{13}$C, $^{31}$P, $^{47}$Ti, $^{48}$Ca, and $^{56}$Fe with a sample rate such that each voxel contained a single sampling of each element. Quantitative data for iron and calcium was obtained by representative ablation of matrix-matched tissue standards. Standards and unknown samples were normalized to background values. Quantitative data was reformatted and scaled in Matlab R2016b (Mathworks; Natick, Massachusetts, USA) for visual analysis.

3.3.7 Histology:

Immunohistochemical stains were prepared as described previously. In brief, sections were cut from paraffin blocks, plated on clean glass slides, and placed in 50°C incubator for 15 minutes to better adhere tissue to slides. Tissue was rehydrated with in stepwise xylene, ethanol, and water rinses. Slides were placed in boiling hot 10mM citrate buffer (pH 6.0) for 15 minutes and allowed to come to room temperature. Slides were then rinsed in dH$_2$O and blocked in methanol containing 3% H$_2$O$_2$ for 20 minutes. Tissue was then rinsed in 7.4 pH PBS three times and blocked in 2% milk in PBS for one hour. Sections were then stained overnight at 4°C in a humidity chamber with a cocktail of 1% milk, 0.1% Triton-X and one of three different primary antibodies: anti-A$\beta_{42}$ for fibrillar peptides (1/2000; Rabbit; Abcam ab39377), anti-A$\beta_{40}$ (1/1000; Rabbit; Abcam ab110888), IBA1 (1/1000; Rabbit; Wako 016-20001). The next morning slides were rinsed in 1% milk and three PBS washes. Tissue was incubated with 1:200 Vectastain Elite anti-rabbit or anti-mouse HRP conjugated secondary antibody in 1% milk and 0.1% Triton-X for one hour at room temperature. Tissue was then rinsed in PBS three times and counterstained with Vectastain ABC solution for one hour. Tissue was rinsed three times and HRP brown
reaction was performed with momentary addition of Vector DAB substrate kit solution. Tissue was rinsed with dH$_2$O and dehydrated with stepwise ethanol and xylene rinses. Slides were cover-slipped with non-aqueous mounting media and no.2 glass cover slides.

Modified Perl’s staining was adapted from methods previously described on 5µm brain sections. Briefly, tissue was deparaffinized with two three-minute SafeClear (xylene substitute) rinses and rinsed twice with PBS pH 7.4. Rinsing in lower pH conditions alters iron staining, so physiological pH buffered PBS was used. Iron retrieval was performed with a cocktail of 1% potassium ferrocyanide tri-hydrate (Fisher Scientific P236)/5% polyvinylpyrrolidone (Fisher Bioreagents BP431)/0.05N HCL for 60 minutes. Tissue was quickly rinsed twice with PBS pH 7.4 and placed in methanol containing 0.3% H$_2$O$_2$ for 75 minutes. Tissue was quickly rinsed in PBS twice and stained with cocktail of 0.025% 3,3’-Diaminobenzidine (Sigma-Aldrich D5637)/0.12% H$_2$O$_2$/0.01M pH7.4 tris buffer for 10 minutes. Tissue was dehydrated and cover slipped in the same manner described above.

All slides were imaged with Aperio AT2 Leica slide scanner with 40x objective and white balanced to standardize background staining. Virtual slides were randomized by dietary group and a trained semi-blinded viewer selected a representative brain section by stain and group. Section images were viewed and collected using Aperio ImageScope software.

3.3.8 ROI Selection and Analysis:

ROIs were drawn over 2x micrograms from 40x virtual images and LA-ICP-MS metal maps by a trained semi-blinded experimentalist in the ImageJ (FIJI, v1.51p) ROI Manager. The Allen Brain sagittal mouse p56 atlas was utilized to determine the boundaries of brain regions: isocortex, olfactory area, hippocampal formation, cerebral nuclei, interbrain, midbrain, hindbrain, and cerebellum. $^{31}$P counts and $^{48}$Ca concentration were also utilized to help
distinguish gray and WM boundaries for LA-ICP-MS images (Fig. 3-4.2). $^{31}$P counts and $^{48}$Ca concentration were also utilized to help distinguish gray and WM boundaries for LA-ICP-MS images (Fig. 3-4.2). Phosphorus is more concentrated in WM$^{29}$ and calcium is more concentrated in gray matter$^{30}$. Artifacts such as, folds, holes, or dust, were removed from ROIs prior to analysis.

For Aβ$\text{\textsubscript{42}}$ analysis, histological images were collected at 2x, converted to 16-bit, and binarized using the left histogram tail (Fig 3-6.3); adapted from instruction protocols$^{31,32}$. Automatic histogram selection within ImageJ (eg “MaxEntropy”) was too variable so this was manually selected to determine plaque load. ROI masks were applied to the binarized image, careful to remove noise and artifacts. Analyzed particles were thresholded with a circularity $>0.1$ mask to reduce edge effects and particle size $>10$ to remove noise. Object size and percent area were output, converting pixel size to a metric value.

For IBA1 morphological analysis, histological images were collected at 10x within an ROI, converted to 8-bit, binarized with the binary function, and analyzed particles were thresholded $>10$ to remove noise. Roundness distribution values were collected in a similar manner as previously described$^{33}$ and was calculated in ImageJ as $(4\times\text{area})/(\pi \times \text{major axis}^2)$. Differences between group histogram bins were determined by ANOVA. Output masks were skeletonized and branch length was determined for each object containing one or more junction. This analysis was adapted calculate and output brain number and branch length for all objects with more than one junction in a similar manner as previously described$^{334}$. Differences were determined by ANOVA with posthoc tests in SPSS.
3.3.9 Statistics and Illustration:

SPSS 22 was utilized for statistical analyses. Outliers were removed if they were more than three times beyond the interquartile range. Variance homogeneity was assessed with Levene’s test of equality. ANOVA was used to assess differences between dietary groups. We utilized a LSD posthoc test to determine group differences when variance was homogenous and Dunett T3 posthoc test when variance was inhomogeneous. A Wilk’s partial eta squared test was used to determine the influence of diet, timepoint, strain, and sex on iron measures. Error bars illustrate standard error mean of the sample, and differences were considered different with a p-value <0.05. All graphs and figures were made with DataGraph 4.3 and Adobe Illustrator CS2.

3.4 Results

3.4.1 TMHF present in blood and brain:

3,5,5-trimethyl-hexanoyl-ferrocene was identified in mouse serum and brain tissue. Figure 3-2.1 depicts a chromatogram and spectrum measured from our standard and animal samples. The chromatogram presents with large peak at 327.1 Da and a smaller peak at 141.2 Da, illustrating TMHF and the 3,5,5-trimethyl-hexanoyl fragment, respectively. Elution time for TMHF in standard and samples were found at 2.53 minutes (Fig 3-2.2). Average TMHF in serum was 0.619ng/ml and decreased upon fasting, measuring 0.223 ng/ml after 10 hours (Figure 3-2.3). In the brain, TMHF measured highest in the olfactory bulb in both males and female mice at 1.40ppb. Female isocortex contained 1.04ppb TMHF while male isocortex contained 0.728ppm. The isocortex was the only brain region to show a sex difference with a p-value ≤0.01 (Fig 3-2.4).
Figure 3-2

1) Graph showing intensity (cps) vs. m/z, Da with peaks at 71.1, 141.2, and 327.1.

2) Chromatograms with peaks labeled as 2.53 Standard and 2.53 Sample.

3) Graph showing ng TMHF/mL serum (ppb) vs. Hours Fasted with a peak at 6 hours.

4) Bar chart showing ng TMHF/g wet tissue (ppb) for different brain regions:
- olfactory bulb
- cerebellum and hindbrain
- isocortex
- subcortical nuclei

Legend:
- Red bars: brain TMHF
  - n = 27, 25, **
Figure 3-2. HPLC of TMHF in serum and brain samples

1) identification of TMHF mass-charge peaks in standard and unknown samples. 327.1 peak illustrates that we can identify full TMHF compound in standard sample, and 141.2 illustrates the 3,5,5-trimethylhexanoyl fragment. Sample tracings of the standard and serum sample illustrating the 2.53 minute elution time of the TMHF compound. Area of peak (gray) was used to quantitate standard curve and unknown sample concentration. 2) TMHF concentration in male and female mice (combined) serum during 10-hour fast. Curve shows that TMHF concentration decreases over time. 3) TMHF concentration after 12-hour fast in male and female mice brain regions: olfactory bulb, cerebellum and hindbrain, isocortex, and subcortical nuclei. The olfactory bulb contained the highest concentration of TMHF. Female mice had a higher concentration of TMHF in the isocortex. Significant difference between sex illustrated: ** $p$-value $\leq 0.01$. 
3.4.2 **TMHF and iron-deficient diet alter RBC and WBC values:**

The normal iron diet WBC significantly increases from two to six months, and the TMHF diet causes WBC to significantly increase from six to twelve months (Table 3-1). Deficient diet significantly decreased RBC at twelve months compared to normal diet. Average HGB and HCT was lower in the deficient group and higher the TMHF at every timepoint compared to normal diet, but the difference was not significant. MCV was significantly lower in the deficient group at every timepoint, and significantly increased overtime. MCV was significantly higher in the TMHF group at six and twelve months compared to normal diet. Platelet volume was significantly elevated in the deficient and TMHF diets at two months compared to normal diet.

3.4.3 **TMHF increases brain iron and iron-deficient diet decreases brain iron:**

Multivariate analysis across 2-, 6-, and 12-months sample times illustrated that diet had the greatest influence on liver iron concentration. Wilk’s partial eta squared for diet sex, and strain were 0.902, 0.187 and 0.175, respectively, meaning that over 90% of the iron difference was influenced by diet. Thus, cohorts were compared based on time and diet because these factors influenced liver iron the most (Fig 3-3.1). Levene’s equality of variance test was not significant for all time points, so a Dunnett’s T3 test was utilized to measure significant differences between groups. The mean liver iron concentrations after two months of diet were 21.7 ppm (Deficient), 94.1 ppm (Normal), and 1420 ppm (TMHF) and were significantly different with p-values ≤0.001. The mean liver iron concentrations after six months of diet were 18.2 ppm (Deficient), 174 ppm (Normal), and 2645 ppm (TMHF) and were significantly different from one another with p-values ≤0.001. The mean liver iron concentrations after 12 months of diet were 24.8 ppm (Deficient), 179 ppm (Normal), and 2290 ppm (TMHF) and were significantly different
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Table 3-1. Dietary effects on blood parameters

Parameters selected for analysis: White blood cell count (WBC), Red blood cell count (RBC), Hemoglobin (HGB), Hematocrit, Mean corpuscular volume, Platelet count (PLT). Gender and strain were combined in analysis because there were no significant differences. Lower and upper 95% confident intervals displayed and p-values illustrate within dietary comparison by one-way ANOVA to 2 months and across diet by timepoint. Significantly different than normal iron dietary group: * $p\text{-value} \leq 0.05$, ** $p\text{-value} \leq 0.01$, *** $p\text{-value} \leq 0.001$. Significantly different from within diet 2-month timepoint: # $p\text{-value} \leq 0.05$, ## $p\text{-value} \leq 0.01$, ### $p\text{-value} \leq 0.001$
Figure 3-3. ICP-MS of liver and brain iron at 2, 6, and 12 months

Sex and strains were statistically grouped, and significant differences are illustrated by letter association (a-f). 1) Liver iron concentrations. The deficient dietary livers were significantly lower than the normal and TMHF dietary groups’. The average iron deficient livers contained less than 30 ppm liver at all timepoints. Both the normal and TMHF livers reached an iron concentration plateau at 6 months with an iron concentration of 200 ppm and 2000 ppm respectively. 2) Brain iron concentration. There were no significant differences between dietary groups at 2 months. Significant differences were apparent at 6 months and became more apparent at 12 months. Group significance was determined by \( p\text{-value} \leq 0.05 \).
from one another with p-values ≤0.001. Iron deficient liver iron significantly increased from six to twelve months with a p-value of 0.03. Normal and TMHF liver iron significantly increased from two to six months with p-values ≤0.001.

Multivariate analysis across 2-, 6-, and 12-months sample times illustrated that diet had the greatest influence on brain iron concentration, similarly to what was found in the liver. Wilk’s partial eta squared for diet sex, and strain were 0.612, 0.124 and 0.022, and Levene’s equality of variance test was >0.05. Thus, comparisons were stratified similar to the liver iron analysis (Fig 3-3.2). The mean brain iron concentrations after two months of diet were 10.5 ppm (Deficient), 11.3 ppm (Normal), and 11.0 ppm (TMHF) and were not significantly different with p-values ≤0.05. The mean brain iron concentrations after six months of diet were 11.6ppm (Deficient), 14.1ppm (Normal), and 16.5ppm (TMHF) and were significantly different from one another with p-values ≤0.001. The mean brain iron concentrations after 12 months of diet were 12.8ppm (Deficient), 16.3ppm (Normal), and 20.8ppm (TMHF) and were significantly different from one another with p-values ≤0.001. Each diet resulted in a significant increase in brain iron at each timepoint, however, the difference across time was smaller with deficient iron diet and larger with TMHF diet.

3.4.4.4 Iron deposits in focal areas of the brain during age:

Figure 3-4.1 displays laser ablation iron concentration maps of NL-F and NL-G-F brains at 6 and 12 months and illustrates progressive regional iron load with different iron diets. Six-month brain CA1 pyramidal neurons and olfactory bulb fiber tracts and olfactory bulb cellular layers contain the highest level of iron and don’t appear to be different across dietary groups, however, there appear to be higher levels in the NL-F group. At 6-months TMHF appears to increase brain iron within the stratum of CA1, subiculum, thalamic nuclei, basal ganglia,
Figure 3-4. LA-ICP-MS map of sagittal brain iron

1) Mouse brains of deficient, normal, and TMHF diet animals contain a distinct and heterogenous distribution of iron that increases with time. All dietary groups contain a relatively high iron concentration within the glomerular, mitral, inner plexiform and granular layers of the olfactory bulb and pyramidal layer of CA1 and CA2 regions of the hippocampus. However, there was an apparent dietary increase in the olfactory granule layer, hippocampal dentate granule layer, subiculum, z substantia innominate, choroid plexus, bed nuclei of the stria terminalis, thalamic nuclei, midbrain colliculi, cerebellar nuclei and molecular layers. These areas showed increased iron concentration from 6 to 12 months, but the effect was magnified with TMHF dietary iron. NL-G-F brains contained speckling of high iron within the cortex, which was magnified in the TMHF diets at 6 and 12 months. The NL-F brains showed speckling at 12 months. 2) Calcium, phosphorous and iron maps of 12 month TMHF fed NL-F mouse. Yellow dotted lines illustrate demarcation of corpus callosum, fornix, and anterior commissure. Red dotted line illustrates that the outer edge of the anterior commissure contains a higher level of iron. The solid thin yellow lines in the phosphorous map illustrate ROIs selected for analysis. Representative ROI histogram of olfactory area iron concentration. 3) Average iron concentration and distribution for single mouse by strain, sex, diet, age, and ROI: isocortex, olfactory area, hippocampal area, cerebral nuclei, interbrain, midbrain, hindbrain, and cerebellum. Highest iron concentration was observed in the olfactory area and hippocampal area. Iron concentration histograms by region illustrate a longer spread of high concentration by age, region, strain, and sex (n=1/group).
substantia nigra, cerebellar molecular layers, and choroid plexus. Magnified areas within the NL-G-F TMHF brain illustrate speckling of high iron within the cortex, CA1 and subiculum indicative of high focal iron deposition.

Twelve-month brain tissue contain higher levels of iron within the olfactory bulb, hippocampus, midbrain colliculi, thalamic nuclei, cerebellum, fornix, and the splenium of the corpus callosum. These same areas also appear to increase brain iron with different iron diets. More concentrated iron speckling appears in 12-month NL-G-F mouse fed TMHF relative to the 6-month animal. Speckling appeared within the thalamus, basal ganglia, CA1, subiculum, and frontal cortex, indicative of further abnormal iron deposition within those area. TMHF also increases iron within choroid plexus and WM regions, such as the caudal head of the corpus callosum, outer ring of the anterior commissure, and fornix.

Figure 3-4.2 illustrates comparative calcium, phosphorus, and iron maps of mouse brain (12M NL-F fed TMHF). Calcium concentration is lower in large WM tracts, such as the corpus callosum and anterior commissure, relative to gray matter regions, such as granule layers of the cerebellum and hippocampal neuronal layers. Phosphorous levels are highest within borders between gray matter and WM and is highest in order of granule layer interface of the cerebellum, neuronal layers of the hippocamps, cellular layers of the olfactory bulb, and layers 2/6 of the isocortex. Iron levels are also highest within the outer layers of the anterior commissure. Figure 3-4.2 illustrates ROI selection within an iron map for regional quantification. The histogram in the lower panel displays the bimodal distribution of iron within cellular and fiber tracts within the olfactory bulb.

Figure 3-4.3 displays brain regional iron differences between strains, diets, and sexes. Iron histograms in mice fed TMHF for 12-months skew highest within the isocortex,
hippocampal area, olfacatory area, and cerebral nuclei. This means that portions of these brain regions contain heterogeneously high concentrations of iron that are elevated after chronic TMHF feeding. The olfactory bulb nerve and fibers tracts of the 6-month and 12-month TMHF fed animals contained iron that was more concentrated than our highest standard, 90 ppm. Other portions of normal brains, such as the hindbrain, midbrain, and interbrain have narrower distributions, suggesting that iron distribution is more homogenous.

3.4.5 TMHF increases amyloid plaque iron deposition:

Figure 3-5.1 illustrates various iron stained brain regions of 12-month fed NL-G-F mice. Iron deposits (black arrows) are apparent in the cortex, hippocampus, thalamus, basal ganglia. These iron deposits take the shape of amyloid plaques with a 20-50 µm diameter. Some of these iron deposits appear diffuse, while others appear like cored plaques with a dense center and diffuse halo. These dense cored iron-laden plaques appear more within the TMHF cortex, while the diffuse and faint iron deposits appear in the iron-deficient cortex and thalamus. The TMHF diet appears to increase staining of WM tracts, such as the outer ring of the anterior commissure (red arrows) within the basal ganglia, the caudal corpus callosum, and fiber tracts within the thalamus. All diets show iron-cored plaques within CA1 and alveus of the subiculum, however, they appear darker and more numerous within the TMHF hippocampus. These cored plaques are also numerous adjacent to darkly stained fiber tracts of the thalamus in the TMHF brain. Iron staining within the choroid plexus is also higher within increasing available dietary iron. These findings parallel what was observed in the LA-ICP-MS analysis.

Figure 3-5.2 illustrates Perl’s stain at higher magnification at 6 months as well as 12 months to depict the presence of cellular iron distribution. Black arrows indicate plaques, which follow the trend previously discussed. Six-month brains also contain iron-laden plaques
Figure 3-5

1. Frontal Cortex, Hippocampus, Thalamus, Basal Ganglia, Choroid Plexus (4th ventricle)

- Deficient
- Normal
- TMHF

2. Frontal Cortex, CA1, Basal Ganglia

- Deficient (6Mo, 12Mo)
- Normal
- TMHF
Figure 3-5. Perl’s iron stain of 12-month NL-G-F brain regions frontal cortex, hippocampus, thalamus, basal ganglia, choroid plexus (4th ventricle)

TMHF fed animals had more iron-laden plaques in the frontal cortex, hippocampus, thalamus and basal ganglia. There is also visibly more staining within the choroid plexus. The deficient fed animals contained less iron-laden plaques in the same brain regions and less iron in the choroid plexus. Black arrows indicate iron-laden plaque and red arrows indicate iron staining around the outer layer of the anterior commissure within the anterior commissure. Scale bar is 200µm. 2) Perl’s stain illustrates cellular iron inclusion within apparent microglial cells within CA1 at 6 months and frontal cortex at 12 months in NL-G-F. Iron staining increases with increasing iron diet within plaques, microglia, oligodendrocytes and WM. Black arrows indicate iron-laden plaque and white arrows heads indicate iron-laden microglia. Scale bar is 50µm
following our discussed dietary effect, however iron within these plaques appear more diffuse, resembling more primitive plaques than cored mature plaques. White arrow heads point at deeply stained microglial structures. These features are only present in hippocampus at six months. At 12 months, they are more present in CA1 and the frontal cortex, where TMHF appears to increase their presence. The data illustrate increased iron occlusions within oligodendrocytes of the basal ganglia in animals that contained higher brain iron.

3.4.6 Aβ42 plaques are denser with TMHF diet

Aβ42 fibrillar stains in multiple brain regions of 12-month fed NL-F and NL-G-F mice (Fig. 3-6.1). Morphology of amyloid plaques in NL-F mice appeared diffuse and primitive within the cortex. NL-F mice appeared to develop more cored-plaques within the stratum oriens of CA1 and the subiculum of the hippocampus. There was no discernable visible difference in plaque morphology between dietary cohorts in the NL-F strain. NL-G-F mice presented with dense fibrillar Aβ42 staining at 12 months. We found numerous mature cored-plaques within various brain regions including the isocortex, hippocampus and basal ganglia. The hippocampus had dense cored-plaques throughout the CA1 stratum. Deficient iron mice presented with fewer cored plaques and more diffuse plaques as compared to normal iron fed mice. TMHF fed mice appeared to present with more cored plaques within the cortex, hippocampus and basal ganglia.

Regional percent area and size of Aβ42 plaques in the NL-F and NL-G-F mice by brain region: I) isocortex, II) olfactory area, III) hippocampal area, IV) cerebral nuclei, V) interbrain, VI) midbrain, VII) hindbrain, VIII) cerebellum (Figure 3-6.2). The regional numerical order demarcates the temporal order of plaque deposition within the NL-G-F brain over 12 months. The NL-F mice only had Aβ42 plaques within the isocortex, olfactory bulb and hippocampus at 12-months. Plaque load within these regions of the NL-F were minimal at 6 months, so this time
### Figure 3-6

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<th>Frontal Cortex</th>
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**Deficient**

**Normal**

**TMH-F**

#### 2

- **I)**
  - 0.11% TMHF
  - Normal Iron
  - Iron Deficient

- **II)**
  - Average plaque % Area

- **III)**
  - Average plaque size (um²)

- **IV)**
  - Months on diets

#### 3

- **i)** ROI Selection
- **ii)** Acquired Image
- **iii)** Gray Scaled (16-Bit)
- **iv)** Tail
- **v)** Binarized Image
- **vi)** Particle Threshold

**Histogram selection**

**Average plaque % Area**

**Average plaque size (um²)**

**Months on diets**
Figure 3-6. Aβ42 stain

1) Aβ42 stain of 12 month NL-G-F (n=6/dietary group) and NL-F (n=3/dietary group/sex) brain regions: frontal cortex, hippocampus, and basal ganglia. Scale bar is 200µm 2) Quantitative % Area and plaque size by brain region: I Isocortex; II Olfactory Bulb; III Hippocampal Area; VI Cerebral Nuclei; V Interbrain; VI Midbrain; VII Hindbrain; VIII Cerebellum. NL-F 12-month data is presented with males and females. NL-G-F 2, 6 and 12 month data is presented with grouped males and females. 3) Illustration of quantitative image processing in ImageJ: (i) ROI selections; (ii) Raw image; (iii) converted 16-bit image; (iv) histogram threshold to select for plaques; (v) binarized image; (vi) binarized image after particle thresholding to reduce noise and border artifact. * p-value ≤0.05; ** p-value ≤0.01; *** p-value ≤0.001
point was not analyzed. NL-F females averaged more Aβ42 staining within the isocortex and olfactory bulb across all dietary cohorts, but was only significantly different in the isocortex of the TMHF group. There was no Aβ42 load difference among NL-F dietary groups. Plaque size ranged from 150-350μm² within the NL-F strain, and was also not different among dietary cohorts.

Since Aβ42 plaque load developed as early as two months within the NL-G-F mouse we could evaluate temporal changes. There was not a sex difference, so male and female data were combined for the NL-G-F mice. All brain regions evaluated had a significant Aβ42 load increase from two to six months; the midbrain, hindbrain and cerebellum were the only regions to show a significant increase from six to 12 months. Aβ42 size plateaued in regions with heavy amyloid load. Plaque size within the interbrain, midbrain, hindbrain and cerebellum increased from two to six months, plateauing at an average 100-150 μm². The cerebellum, which developed plaques last was the only brain region to show increased plaque size from 6 to 12 months. Brain regions that developed plaques earlier, such as the isocortex, hippocampus and cerebral nuclei, significantly decreased in average size by 10-20% from 6 to 12 months. Experimental iron diets significantly increased brain Aβ42 load or size within the isocortex, olfactory area, hippocampal area and cerebral nuclei at the two-month timepoint. Experimental iron diets did not affect brain Aβ42 saturation or size at later timepoints.

3.4.7 Aβ40 plaque number decreases with increasing brain iron:

Figure 3-7 illustrated that Aβ40 plaques are visible in NL-G-F mouse as early as the two-month dietary timepoint. The Aβ40 plaque appears as a diffuse cluster throughout the cortical and subcortical regions 20-50 μm in diameter. At two months there is no apparent dietary
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<td>6 Months</td>
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Figure 3-7. Aβ₄₀ stain

Aβ₄₀ stain of 2, 6 and 12- month NL-G-F brain regions: frontal cortex, basal ganglia. Aβ₄₀ plaques appear oligomeric with granulized clusters. Aβ₄₀ plaque load and size increases in the frontal cortex from two to six months and decreases from six to 12 months. Aβ₄₀ plaque load is visibly greater in the deficient diet group and absent in the TMHF group at 12 months. Within the basal ganglia, Aβ₄₀ plaques initially forms around the gray-WM boundary of the anterior commissure. At 12 months, Aβ₄₀ is distributed more evenly through the bed nuclei of the stria terminalis and other portions of the basal ganglia. The white dotted line indicates the border of the anterior commissure. Black arrows indicate Aβ₄₀ plaque staining. Scale bar is 200µm.
influence on Aβ40 plaque load. The plaques are abundant and round within the cortex, and a few oblong clusters are present around the edge of the anterior commissure in the basal ganglia.

There is an increasing load of Aβ40 positive plaques within the cortex and subcortical regions at six months with diameters ranging from 20-80 µm in diameter. Within the cortex there is a visible dietary cohort difference; plaques appear with darker granule deposits within the deficient and normal iron diets and more diffuse in the TMHF diet. At six months, there are plaque throughout the basal ganglia. However, there are less plaques around the anterior commissure. At 12 months, plaque load and size in the cortex is lower than previous timepoints, and there appears to be a dietary plaque load difference. The deficient group has the highest plaque load and the TMHF group show no visible plaques. There are no visible plaques within the TMHF group’s brain. Aβ40 increases in the basal ganglia from 6 to 12 months; the dietary differences illustrated in the cortex are also present in the basal ganglia.

3.4.8 Microglial become more phagocytic with increasing brain iron:

Figure 3-8.1 illustrates IBA1+ cells in the frontal cortex, CA1 and basal ganglia. Among all dietary groups, increasing microglia clustering occurs in regions of the brain with increasing amyloid deposition. The most evident difference between dietary groups is the dearborization of microglial cells in the TMHF group. Six-month fed TMHF mice present with less endpoint processes with thicker somal processes. These cells appear to be hypertrophic with wider cell bodies. At 12-months, there is increased microglial clustering in addition to decreased arborization in all dietary groups. TMHF appears to increase the amoeboid appearance of microglial cells, since they are rounder with shorter processes.

Figure 3-8.2 depicts roundness of the IBA1+ cells in the brains of 6- and 12-month brains. Differences between dietary groups represented by symbol. At six months, microglial
Figure 3-8

1. Frontal Cortex - Normal and Deficient at 6Mo and 12Mo, CA1 - Normal and Deficient at 6Mo and 12Mo, Basal Ganglia - Normal and Deficient at 6Mo and 12Mo.

2. Graphs showing roundness distribution over 6 Months and 12 Months for Frontal Cortex, Hippocampus, and Basal Ganglia with different conditions: Deficient, Normal Iron, and 0.11% TMHF.

3. Bar charts showing branch length (μm) over 6 Months and 12 Months for different conditions: 0.11% TMHF, Normal Iron, and Iron Deficient.
**Figure 3-8. IBA1 histology and quantitative analysis**

1) IBA1 and stain of 6 and 12 month NL-G-F brain regions: frontal cortex; hippocampal CA1, and basal ganglia. IBA1+ microglial cells in mice fed TMHF present with a reduced arbor ramification and thickened processes as early as 6 months in observed regions. Scale bar is 50µm. 2) IBA1+ microglia roundness by diet and brain region: frontal cortex, hippocampus, and basal ganglia. TMHF fed animals have rounder cells, and the effect is most significant at 6 months. Differences between dietary groups represented by symbol: * = p-value<0.05 for normal to TMHF; # p-value<0.05 for deficient to TMHF; ^ p-value<0.05 for deficient to normal. 3) Branch length is reduced in TMHF diet in all brain regions and timepoints observed, and the effects is most significant at 6 months (n=6/group). *** = p-value<0.001
cells are rounder in the frontal cortex, hippocampus, and basal ganglia of animals fed TMHF. There is a significant difference between difference in the frontal cortex and hippocampus of deficient mice and TMHF mice, and there is a significant difference between normal and TMHF mice in the basal ganglia. However, TMHF IBA1+ cells trend rounder in all regions. At twelve months, there are less significant differences between dietary groups. The frontal cortex and hippocampus contain cells that are significantly different between TMHF and normal iron groups, but the effect size is smaller.

Figure 3-8.3 depicts IBA1+ microglia branch length over time between dietary groups. IBA1+ positive branch length is shortest in the cortex, hippocampus and basal ganglia of TMHF fed animals at 6- and 12-month animals. Branch length is shorter in the hippocampus and basal ganglia of deficient mice at 6 months relative to normal iron mice. The same comparison is significant in the basal ganglia at 12 months.

3.5 Discussion

TMHF elevated brain iron in a region-specific manner and increases amyloid plaque iron load. In addition, TMHF diet increased microglia roundness and reduces branch length in the frontal cortex, hippocampus, and basal ganglia. Although Aβ42 plaque morphology appeared more punctate in high brain iron conditions, brain iron levels did not affect plaque load or size of fibrillar Aβ42 plaques. Deficiency increased Aβ40 plaques, while TMHF reduced and eliminated Aβ40 plaques at 12-months. The data illustrate for the first time that dietary TMHF is found with the blood and brain, demonstrating gut and blood brain barrier penetrance. We observe that MCV is a more sensitive measure to iron deficiency and iron elevation than HGB and HCT.

The LA-ICP-MS data highlight that regions of the TMHF hippocampus and frontal cortex have focally high levels of brain iron, and Perl’s stains illustrate that these same regions
contain iron-laden plaques. Hare et al. found that AD brains have more iron infiltration of frontal gray matter. Our data demonstrate punctate iron deposition within gray matter supporting elevated iron infiltration and Aβ plaque load. Areas of the medial prefrontal cortex are shown to have high iron-amyloid coupling, and this coupling is linked to cognitive deficit in MCI patients. Our findings support the hypothesis that iron associated plaque load facilitates oxidative stress. Previous findings support the idea that iron loading plaques is associated with cognitive decline, while individuals with amyloid load without increased iron deposition are cognitively normal. The medial prefrontal cortex of humans may be especially burdened by iron-laden amyloid as it is adjacent to the anterior position to the corpus callosum genu, a region especially vulnerable to age-linked demyelination and oligodendrocyte stress. Myelin loss may lead to subsequent iron release, which would facilitate amyloid iron loading. We show that regions of the mouse brain with high WM iron, such as the caudal corpus callosum, anterior commissure, thalamus, and basal ganglia also develop iron-laden plaques. Inconsistencies in amyloid-iron staining from previous rodent work may be due to mouse strain phenotypic differences or variation in histological iron staining. Nonetheless, we show that mice on the TMHF diet developed increased WM iron, iron-laden plaques, and microglial iron deposits, mimicking human AD.

Amyloid iron and tissue iron level may impact the function and morphology of IBA1+ microglia. Healy et al. showed decreased IBA1+ processes in organotypic culture after administering ferrocene. Our work confirms that TMHF decreases microglial branch length, which indicates microglial activation. IBA1+ cells also appear to be more round in regions of the brain that have elevated brain iron at six months, such as the basal ganglia. This indicates that the microglial cells are assuming a more phagocytic morphology in the presence of iron. At
twelve-months, the effect of diet on microglia morphology is less; cells are less round with longer and wider branches. The increased number of aggregating microglia and activated microglia with age may make morphological differences between different brain iron states more difficult to detect. Elevated amyloid burden and age increase the level of dystrophic microglia and amoeboid cells, and both aging and AD lead to microglia process retraction. Dearborization and process retraction may influence average process length in inverse ways. Our results suggest that dearborization from a ramified state to amoeboid state decreases process length, while activated hypertrophic microglia increase average process length. Iron-laden plaques and increased brain iron promote more amoeboid microglial cells during early stages of amyloid deposition that may alter AD pathology.

We found that altered brain iron levels do not change fibrillar Aβ42 plaque levels, however, increasing brain iron does decrease Aβ40 plaque deposition. These data run counter to several recent studies in other animal models that suggest increased brain iron elevates and Aβ levels. There are several differences between these studies and our study. We utilized a lipophilic iron compound in an AD transgenic mouse model that normally expresses endogenous levels of APP, and we histologically investigated plaque load and morphology. Based on previous evidence, it is likely that the APP peptide is increased with higher iron levels, as it is regulated by an IRE. However, fibrillar Aβ42 plaque level may not differ if beta secretase activity and polymerizing kinetics are saturated. Changes in Aβ40 plaque load may explain morphological differences of Aβ42 plaques. Increasing Aβ42/40 peptide ratio in plaques is associated with human-like cored plaques because it is more easily incorporated into hydrophobic fibrils. Elevated plaque Aβ42/40 is theorized to be more neurotoxic as it increases the hydrophobicity of Aβ oligomers. Reduction of Aβ40 content in TMHF plaques may
make them more neurotoxic. The role that iron plays in $A\beta_{40}$ aggregation and clearance is still unknown. Since $A\beta_{40}$ is more easily cleared from the brain$^{346}$, iron may enhance that function. $Fe^{2+}$ may also bind to $A\beta_{40}$, inducing an N-terminal fold, which retards secondary nucleation$^{347}$. Ferritin levels can also alter gamma secretase activity, so elevated $A\beta_{42}/40$ may be function of altered enzymatic cleavage$^{348}$. Increased microglial activation under high iron conditions may also facilitate amyloid peptide degradation$^{275}$.

$A\beta_{42}$ plaque levels formed rapidly in the NL-G-F and slowly in the NL-F mice. This observation supports previous findings$^{323}$. We also found increased amyloid plaque burden in female NL-F mice but not in female NL-G-F, which was not reported in previous findings that only evaluated mice at 18-months$^{349}$. The NL-F mouse $A\beta_{42}$ plaque is larger and more diffuse, while the NL-G-F plaque is usually smaller with a cored senile morphology. The arctic mutation facilitates faster and denser polymerization$^{350}$, which may cause these morphological and plaque load differences between the two strains. Our thorough investigation of plaque load and size in both models illustrates that regional plaque deposition occurs in a temporally demarcated way. $A\beta_{40}$, which is more soluble, may travel more easily via perivascular clearance mechanisms while $A\beta_{42}$ may not. In addition, vascular changes from gray to WM may impede these clearance mechanism, which may explain seeding and large cored plaques adjacent to WM structures. Brain regions that become $A\beta$ plaque saturated earlier reduce in size later, indicating that either older plaques seed smaller plaques or active degradation is occurring after plaque formation. Both the NL-F and NL-G-F mouse presented with more cored iron-laden plaques within CA3, CA1, and subiculum of the hippocampus. These are regions of the brain that are susceptible to neuronal loss within the AD brain$^{351}$.
We found that TMHF is present in the serum and brain within a brief period of *ad libitum* consumption. Blood TMHF concentration declines with fasting, suggesting that it is quickly metabolized and stored within tissues. TMHF has been shown to be metabolized in the liver by phenobarbital-inducible cytochrome P-450 following Michaelis-Menten kinetics\(^3\). A variety of P-450 enzymes found in the liver are also found differentially distributed throughout the rodent brain by region\(^3\). P450 may also function differently by tissue type and sex, which may explain why TMHF levels are higher in the olfactory bulb and female mice.

Blood measures indicate that mean corpuscular volume (MCV) is most readily and profoundly affected by iron diet differences. Iron deficiency diet leads to microcytic red blood cell while TMHF leads macrocytic red blood cells. Increasing WBC counts in the TMHF later in the study may indicate the onset of infection or inflammation. This supports the notion that increasing free iron may predispose the body to infection and inflammation. Recent research has shown that acute oral administration of iron elevates bacterial growth within human serum\(^3\). We did not correlate blood values with amyloid load in the blood or brain, and the relationship between anemia and Alzheimer’s is still mixed. Work by Chen *et al* showed that AD patients have increased MCV, HGB, RBC, PLT, and WBC compared to healthy controls\(^3\), while Faux *et al* showed that lower hemoglobin is associated with cognitive impairment and AD\(^3\). Our dietary cohorts alter blood measures, but it is still uncertain if these levels impact the disease course in our mice.

Chronic iron deficiency diet significantly decreases total brain iron and that chronic TMHF diet significantly increases total brain iron after six months of *ad libitum* feeding with a magnified effect at 12 months. These findings imply two key points about brain iron loading. Firstly, the aging brain acquires iron from diet and peripheral tissues throughout the aging
process. Secondly, metabolism of TMHF within the brain may be slower than in the viscera. Although TMHF quickly diffuses into the brain, brain iron is not significantly elevated until six-months of feeding. Gross brain iron concentration between NL-F and NL-G-F brain iron at all timepoints in all dietary conditions appears similar. This suggests that circulating iron affects total brain iron stores more than brain amyloid load and amyloid load does not alter total brain iron level. Thus, vascular health and iron transport in and out of the mouse brain is not altered between our two models. Work by Moon et al reveals that vascular dementia and AD subject have a similar iron increase within the caudate and putamen, suggesting that vascular damage may facilitate heme and ferritin to leak into the brain. Further work will need to be conducted to evaluate how different amyloid load affects iron homeostasis.

These findings should be framed in the context of the literature base and limitations within the study design. The work utilized a familial APP transgenic mouse mutant that rapidly incurs amyloid deposition to observe a human condition that takes decades to develop. The longitudinal course of amyloid deposition and its relation to iron between human AD and the transgenic mouse brain should be taken into account. The number of animals utilized in the study design as well as subsequent sample size limitations should be considered. Several of our analyses showed consistent biological trends without demonstrating mathematical significance. In addition, many of our findings are limited to qualitative histological analyses, further protein analyses is required to support our findings. Future behavioral work will also need to be conducted to observe the interaction of brain iron and amyloid on memory.

With these thoughts in mind, this work indicates elevated brain iron in the aging brain increases iron-laden plaque load. This condition is associated with increased histological Aβ_{42}/Aβ_{40} ratio and denser cored plaques. In support of other work, TMHF elevated iron leads to
microglial deaborization; all features that better mimic human AD Aβ plaques. Thus, elevated brain iron within the knock-in APP mice may better resemble features within the human condition that lead to atrophy and memory loss in AD patients.

3.6 Acknowledgement. We would like to thank the Hare Lab for graciously sharing their matrix matched metal standards and Skyping with us at odd hours of the day to teach us about LA-ICP-MS. The Stoute Lab facilitated our blood parameter analysis by allowing us to use their hematology auto analyzer. We would also like to thank the Cheng Lab and Jean Copper for allowing use to use their clinical slide scanner.
Chapter 4
Iron loading during aging and disease: how, what, when and why

4.1 Introduction

Brain iron accumulation occurs normally in the aging brain. The most rapid elevation occurs between early infancy and adolescence to facilitate the energy demands of myelination and neurotransmitter synthesis (eg. DA). However, after middle-age, mishandling of iron can reduce the cellular energy supply, impair waste clearance, and provoke oxidative stress—leading to early senescence and apoptosis. These cellular changes are represented by phenotypes consistent with a rare diagnosis called neurodegeneration with brain iron accumulation (NBIA). This condition serves as an example of brain iron accumulation without amyloidogenesis. This condition is known to be caused in one of 10 possible inherited mutations that lead to iron accumulation in the basal ganglia, olfactory bulb, and cerebellum resulting in progressive dystonia, spasticity, parkinsonism, neuropsychiatric abnormalities, blindness, and hyposmia. New research suggests that brain iron regulation is facilitated by a host of proteins that are linked to more common neurodegenerative diseases, such as the amyloid precursor protein, which is a progenitor protein in the development of amyloid plaques in AD (AD).

This dissertation explores the effect of dietary iron overload and deficiency in a wildtype control (C57BL6) and human amyloid generating mouse models (NL-F and NL-G-F). In Ch. 2 we chronically fed mice an iron-deficient diet and a novel lipophilic iron compound (3,5,5-trimethylhexanoyl ferrocene; TMHF) to observe how these two conditions altered brain iron. We highlight for the first time that TMHF can be found in the brain and blood of mice after oral
exposure. The work demonstrates that in C57BL6 mice, chronic TMHF administration significantly increased brain iron by 15-35% within the hippocampus, cortex, basal ganglia, and cerebellum; these are areas clinically associated with dysfunction and atrophy in AD and NBIA. Iron deficiency did not affect iron levels in the above brain regions. *In vivo* MRI analysis in wild-type mice revealed that TMHF accelerated and increased iron loading in regions of the basal ganglia, olfactory bulb, substantia nigra, and corpus callosum relative to normal and iron-deficient mice. TMHF was also found to increase WM iron and preserve volume fraction. The WM fraction decreased over time in the normal brain iron condition. Histological analysis illustrates that TMHF increases the iron storage protein, L-ferritin, in the brain to facilitate normal handling of elevated iron. Despite the normative response to sequester toxic free iron, TMHF was also found to elevate the protein markers for oxidative stress, inflammation and gliosis. IBA1+ microglia appeared rounder and phagocytic in the TMHF condition. TMHF fed animals also developed impaired spatial learning. We further explored the role of TMHF and iron-deficiency on the development of AD pathology and brain iron loading.

AD is an age-onset progressive neurodegenerative disease, which leads to profound memory loss and mood dysfunction. The disease manifests with primary extracellular deposition of beta amyloid-Aβ plaques with neuritic microglial inclusions. New histologic evidence suggests that iron dyshomeostasis may increase Aβ peptides, iron-rich plaques, and iron-rich microglia in AD compared to healthy patients. Similar to our C57BL6 study in Ch. 2, Ch.3 reports that TMHF elevated brain iron in the NL-F and NL-G-F mice by 22%. However, iron deficiency significantly decreased brain iron by 21%. These mutant strain differences may highlight the vital role that APP and Aβ play in normal in iron accumulation and clearance in the brain.
We utilized laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) and Perl’s iron stain to illustrate that amyloid plaques stained more intensely for iron in elevated brain iron regions, such as the hippocampus and basal ganglia. The content of iron-rich plaques had a higher $\text{A}\beta_{42/40}$ plaque load and presented with more densely packed core-like features. IBA1+ microglia had shorter branches and rounder bodies in high iron conditions, suggesting an increased phagocytic profile concurrent with amyloid deposition. Microglia can digest amyloid, but oxidative stress and over excitation may prevent these cells from performing their normal housekeeping functions in the brain. Dysfunction of microglia may lead to iron dyshomeostasis.

Specific brain structures, such as the basal ganglia, hippocampus, substantia nigra, olfactory bulb, cerebellum, and corpus callosum within the mouse brain normally sequester iron. These subcortical regions contain cells that are highly integrated with cortical areas and themselves. The metabolic demand of these cells requires them to utilize more iron. In addition, we suggest that synaptic dysfunction in these brain structure may be linked to iron deregulation and accumulation. We found that elevated brain iron increases iron deposition within amyloid plaques, the number of phagocytic microglia and impairs spatial learning. At the same time, elevated brain iron may also support the metabolic demand of oligodendrocytes to better support myelin integrity throughout the brain. In addition, amyloid pathology may impair proper iron loading into the mouse brain during low iron conditions. Our model system illuminates that brain iron storage is pooled into specific brain structures. Like a river that channels water and nutrients in a given direction and rate, the brain channels iron along a given path. Flooding the brain with iron can erode and weaken the element’s storage and transportation mechanisms, but iron is still directed along the same path. Boulder-like amyloid plaques can even inhibit iron flow, and they can be shaped by the flow of iron through them.
4.2 Implication for TMHF distribution and metabolism in a mouse brain

The lipophilicity and small size, less than 400Da\textsuperscript{360}, of TMHF \textsuperscript{361} allows it to diffuse throughout the brain. Three possible mechanisms of accumulation may be taking place: 1) passive absorption into cells that can trap TMHF, 2) Variable TMHF metabolism by cell type that reduces TMHF, or 3) a combination of both.

Passive absorption and diffusion of a molecule through the blood brain barrier is believed to be dependent on the Gibbs adsorption isotherm \textsuperscript{362}, which is reliant on molecular concentration and surface tension. Surface tension is dependent on surface area and chemical potential of a molecule. In addition arterial and capillary flow dynamics can influence drug or small molecule distribution in a heterogenous manner in the brain\textsuperscript{363}. Thus, compounds at high concentration that are small, hydrophobic and uncharged will pass easily through the blood brain barrier (BBB). Once in the brain, TMHF may diffuse through the extracellular space (ECS). This diffusion is affected by a number of properties including volume fraction, geometry, width, local viscosity of the ECS, interaction with cellular surfaces, extracellular matrix, and components of the interstitial fluid \textsuperscript{364}. Basic psychotropic drugs collect more readily in gray matter via the acidic lysosomal trapping, whereas aliphatic-type molecules collect more readily via phospholipid binding. The lipophilic nature (“fat-loving”) of TMHF and its carbon chain may facilitate higher phospholipid binding. Thus, higher concentrations would be found in myelin bundles, which are rich in densely packed phospholipid membranes. The olfactory bulb is a small gray matter structure with two large WM bundles, the anterior and lateral commissure. The high ratio of WM to gray matter in this structure may explain why TMHF is so highly concentrated there.
The concentration of TMHF by brain region may be a combination of accumulation and enzymatic cleavage. As described in Ch 2 and Ch 3, TMHF is thought to be metabolized by cytochrome P450s (CYPs)\textsuperscript{352}. There are six subfamilies recognized as being responsible for most drug metabolism. These include CYPs 1A, 2A, 2B, 2C, 2D, 2E, and 3A\textsuperscript{365}. The density and activity of P450 are much higher in the liver than in the brain, and the liver contains many Kupffer macrophage cells that can store iron in ferritin. This would explain why there is a 10-fold increase of iron in the liver compared to the brain\textsuperscript{353}. There is some evidence to suggest that there are various CYPs within astrocytes and microglia, but there is little evidence to suggest their enzymatic activity. The concentration of TMHF in the brain was higher than in the blood after prolonged fasting, suggesting that metabolism of TMHF is slower in the brain. Regions of the brain that contain a lower concentration of TMHF and increased iron, such as the subcortical nuclei, may be brain regions with relatively high TMHF metabolism. TMHF accumulation and metabolism may indicate acute iron accumulation in the brain, but there may be more powerful forces in play that determine long term iron accumulation and storage, such as metabolic demand and protein mediated accumulation of iron.

4.3 Rationale for iron accumulation with age

It was hypothesized that TMHF iron would be elevated in all brain regions. Instead iron was anatomically elevated in structures that normally acquire iron with age. Our Perl’s stain and LA-ICP-MS data show that TMHF increases iron more readily in the olfactory bulb, basal ganglia, hippocampus, thalamus, midbrain, cerebellum, choroid plexus, and WM areas. These findings support our MRI R\textsubscript{2} analysis. Brain iron may be slowly acquired with age and stored in areas that can utilize it. Iron may also be acquired to supplant and mitigate failing metabolic systems. For example, TH loss associated with iron accumulation, may link iron accumulation
within a neuronal network with dysfunctional dopaminergic activity. Although the mouse
cortical structures are very different from that of a human’s, the subcortical regions share many
anatomical and functional similarities, such as dopaminergic signaling. In addition, the
propensity of human brain cells to accumulate excessive iron with age occurs within these same
anatomical locations\textsuperscript{366}. Thus, understanding why we observe iron increase in a mouse may be
highly translatable to the human brain. We will first describe these regional iron increases as
they may relate to normal aging and disease. Thereafter, we will highlight perfusion and
clearance mechanisms of the brain as they relate to these regions during normal aging versus the
diseased state.

The hippocampal formation, a highly interconnected circuit with several regional
components, plays an essential role in memory. Each subregion contains a unique population of
neurons with distinct molecular and functional properties, which is thought to account for their
unique vulnerability to various diseases. The utility of iron in the hippocampus throughout life is
still unclear. Our iron analysis of C57BL6 and APP mutant mice showed a distinct iron increase
in CA3, CA1 stratum, subiculum, and DG with time and increasing iron diet. There is evidence
that iron deficiency disrupts maturation of synaptic function in CA1, thus early and continuous
iron accumulation there may be necessary for normal synaptic function. CA1 and the proximal
subiculum region are most affected in AD, and this may be due to chronic iron accumulation and
oxidative stress. DG and CA3 dysfunction with normal aging-associated memory impairment\textsuperscript{367}
may be due to iron accumulation that occurs in these regions later in life. Iron accumulation at
dopaminergic terminals within the hippocampus may also facilitate normal function. As such,
increasing iron over time may be a mitigating factor to enhance a circuit that doesn’t work
properly. The hippocampus contains dopaminergic fibers from the midbrain neurons that
facilitate memory performance\textsuperscript{368}. D1 and D2 GABAergic interneurons are found in CA3/CA1 fields and DG\textsuperscript{369}. A feedback mechanism, which is impaired during the aging process, may increase iron accumulation in an attempt to increase DA signaling. One mechanism proposed by Duca \textit{et al.} link $\alpha$-synuclein acetylation with both transferrin and dopamine endocytosis at the synaptic terminal\textsuperscript{370}. Cellular DA excitation is found to increase oxygen consumption without altering cellular excitability or function\textsuperscript{371}. Iron may increase in nearby DA synapses due to sustained acute hypoxic conditions via the HIF response discussed in Ch. 1. HIF expression in the hippocampus linked lasting memory improvement\textsuperscript{132} but also increased BACE1 and A$\beta$ generation\textsuperscript{372}.

The olfactory area is a useful model to study iron accumulation with age and disease because it displays long-life sustained neurogenesis, axonal growth, plasticity, and iron concentration. The olfactory bulb is composed of several cellular layers: glomerular layer, external plexiform layer, mitral layer, internal plexiform layer, and granular layer. These cells integrate and filter afferent olfactory information from unmyelinated olfactory epithelia neurons and send myelinated centripetal projections to the primary olfactory cortex. In both Ch. 2 and Ch.3, we found that mouse olfactory bulb glomerular and granular layer iron concentration rises early in life, exceeding levels found in other parts of the brain, including the substantia nigra. In humans, each large glomerular cell must integrate information from at least 16 olfactory receptor neurons\textsuperscript{373}. There are high concentrations of tyrosine hydroxylase and dopamine in the olfactory bulb\textsuperscript{259} relative to other brain regions. Adult born dopaminergic interneurons within the glomerular layer may play a role in experience-dependent plasticity, thus, iron facilitated DA synthesis may be essential for learning different odors. Failure of this integration system and oxidative stress as a result of chronic high iron may lead to glomerular apoptosis and hyposmia,
which is very common in older age. Dysfunction of the olfactory system may also be an early indicator for cognitive decline in AD\textsuperscript{374}.

The basal ganglia is a group of subcortical nuclei: caudate and putamen (dorsal striatum), ventral striatum, nucleus accumbens and olfactory tubercle (ventral striatum), globus pallidus, ventral pallidum, substantia nigra, and subthalamic nuclei. These regions are interconnected within the cortex, thalamus and brainstem to help regulate voluntary movement, procedural learning and emotion. We found iron to increase with time and TMHF diet in various regions of the basal ganglia including the striatum and pallidum. The basal ganglia contains an abundant number of dopaminergic cells and synaptic inputs. Iron elevation and accumulation within this brain structure is well documented with advancing age and neurodegeneration. A previous report by Hill et al showed that a GABA-ergic inhibitor applied to various regions within the basal ganglia substantially decreased brain iron within a few hours. The study did not target specific GABA-ergic neurons, but many, like the hippocampus, may relay information from dopaminergic projections. Further work to better understand the interaction dopamine in this model would better highlight the rapid and kinetic activity of iron in the brain.

The thalamus is a large gray matter region made up of several subregions: lateral dorsal nucleus, lateral posterior nucleus, ventral anterior nucleus, ventral lateral nucleus, ventral posterior nucleus, ventral intermediate nucleus, ventral posteromedial nucleus, and ventral posterolateral nucleus. These regions are highly connected to the hippocampus, cortex, striatum, amygdala and spinal cord. They serve as a hub to relay sensation, movement, alertness and pain. We found that the aging mouse acquire increasing levels of iron within the paracentral nucleus of the intralaminar nuclei of the dorsal nuclei. The thalamus, like the hippocampus and olfactory bulb, contains robust dopamine innervation that may influence activity of cortical, striatal and
amygdaloid regions in which the thalamus is connected\textsuperscript{375}. The macaque monkey brain also shows a widespread and uneven axonal distribution of dopamine transporter \textsuperscript{375} in similar regions of the thalamus where we saw iron increase in the mouse brain. This elevated brain iron may be responsible for gait ataxia associated with normal aging\textsuperscript{376} and later stages of AD. Although thalamic atrophy occurs very late in AD pathology, early dysfunction there may be linked to memory impairment during the disease process \textsuperscript{377}. In our NL-G-F mice, we also found large plaques in the thalamus with iron inclusions, which occurs later in life\textsuperscript{378}.

The cerebellum is a unique brain structure tucked behind the neocortex. It contains a series of interconnected cells within the cortical molecular layer that help process and relay information to the deep cerebellar nuclei, which helps encode movement information. We found that the molecular layer contains a relatively high amount of iron early in life. TMHF diet and time lead to increased iron accumulation in the molecular layer as well as the deep cerebellar nuclei, while iron deficiency leads to a decrease in iron within the molecular layer. Like other structures described, the molecular layer is a hub of input integration. Although there is less evidence to support dopaminergic innervation in the cerebellum, D1-D3, DAT, and TH are found in the cerebellum \textsuperscript{379}. The cerebellum may also accumulate iron because of where it sits in the brain; the ventral surface borders the \textsuperscript{4}th ventricle, which contains choroid plexus cells and CSF.

Choroid plexus iron elevation with TMHF and age may indicate a passage of peripheral iron into the central nervous system. It is cited that the choroid plexus highly expresses a number of iron transport, ferroxidases, and storage proteins: TfR1, DMT1, Dcytb, FPN, hephaestin, ceruloplasmin, FTH, and FTL\textsuperscript{380}. A\textbeta immunization in a healthy mouse lemur causes iron deposition in the choroid plexus and microhemorrhages\textsuperscript{234}. The same finding has been
found in some preliminary human clinical trials. Thus, APP and Aβ may play an essential role in iron transport into our out of the CSF.

Iron accumulation in NBIA diseases match many of the findings that we see in normal aging and TMHF fed animals. Most mutations that cause NBIA are linked to iron storage, iron utilization, and iron transport. Mishandling of iron may facilitate iron to be transported more freely to areas of the brain that can more easily acquire it. MRI evaluation of NBIA patients shows R₂ increases in the globus pallidus adjacent to regions of high inflammation. There is also corpus callosum thinning and cerebellar atrophy. NBIA is also found to increase olfactory area iron and ferritin, which colocalizes with astrocytes and microglia, and is associated with hyposmia. We found that the iron /FtL ratio was increased with the TMHF diet, which may indicate that iron storage is not as efficient as in normal iron conditions

Receptor mediated diffusion and fluid kinetics may facilitate iron distribution in the brain. The discovery of a para-vascular glymphatic system may help to explain regionally heterogeneous distribution of large and small iron binding proteins; it is important to note the size of certain peptides and peptide complexes: Ferritin complex (450kDa), ferritin (20kDa), Tf (80kDa), and Aβ (4kDa). Recent work by Iliff et al demonstrates this concept with two MRI enhancer compounds: Gd-DTPA (MW 938 Da) and GadoSpin (MW 200kDa). The work illustrates that the compounds traverses the para-vascular pathway through the pituitary (Gd-DTPA=GadoSpin) and pineal recess (Gd-DTPA=GadoSpin) and through the olfactory bulb (Gd-DTPA>GadoSpin), cerebellum (Gd-DTPA>GadoSpin), aqueductal gray (Gd-DTPA>>GadoSpin), and pontine nucleus (Gd-DTPA>>GadoSpin). We observe rapid iron and rapid Aβ₄₂ deposition near the pineal recess in areas, such as the superior and inferior colliculi, and the olfactory bulb. There is rapid iron accumulation and slow Aβ₄₂ deposition in
the cerebellum, and slow iron and Aβ$_{42}$ accumulation in areas near the aqueductal gray, such as the midbrain reticular nucleus. Distribution of Aβ and small iron-binding proteins may traverse this pulsatile stream, giving rise to heterogeneous iron and Aβ plaque distributions.

The link between iron deficiency and AD is poorly understood and eating habits of AD patients can confound the causative or associative role that iron deficiency may play in AD progression. We observed that an iron deficient diet lowered brain in both the NL-F and NL-G-F mice relative to C57BL6 mice. It is known that strain differences can affect brain iron$^{383}$. The APP mice are also on a C57BL6 background, so the iron brain deficiency may be due to the APP variant. Since the familial mutation is cleaved more frequently at the cell surface, it may not be able to fulfill its normal role to transport iron. The NL-G-F also appears to have subtle and regional iron reduction relative to the NL-F mouse. Since it is believed that he arctic mutation increases APP reabsorption at the cell surface, differences in APP iron transport may be magnified.

4.4 Implication for normal and experimental TMHF WM alterations

In Ch.2 we found that $R_2$ declined in the distal portions of the genu of the corpus callosum in normal aging. We describe this decrease as a loss of compact iron or ferritin. The bulk of myelin iron is contained within oligodendrocytes, which require iron to facilitate proper mitochondrial function, proliferation and differentiation, growth, and myelination. Oligodendrocyte inclusions are associated with swelling and myelin turnover$^{384}$. During early life, axonal myelination sheaths become thicker due to ongoing lamelle wrappings, however, later in life these wrappings become thinner and more vulnerable to damage. Areas of the brain that myelinate later, such as the anterior corpus callosum and frontal cortex, contain more of
these thin wrappings. Our findings support the theory that later myelinating regions are more susceptible to loss later in life.

We also demonstrate VBM WM fraction to decrease with aging, and that this decrease is attenuated with TMHF. We utilized 3D-T2 single echo scan to make our VBM probability maps, thus, our measure may be a mixture of iron, cellular compactness, and myelin density. Since we observed increased iron with time using our LA-ICP-MS and Perl’s stain, it is unlikely the WM fraction is decreasing due to decreasing iron content. Rather, there may not be enough iron in these WM structures to facilitate proper (re)myelination during the aging process. TMHF may increase iron’s availability to proliferating and differentiating oligodendrocytes in order to facilitate these processes.

Since WM is contains a high concentration of iron it may serve as an iron reserve during aging. This could account for WM loss and regionally specific iron elevation in a normal brain. DA dyshomeostasis and cellular metabolic deficit may facilitate WM breakdown and iron release during the normal aging process, which may in turn impede myelination. VBM analysis of the normal aging brain reveals profound WM loss in the frontal cortex and substantia nigra. These areas contain or are adjacent to large clusters of DA neurons.

Increased WM loss during aging may propagate free iron and contribute to AD Aβ deposition\(^{385}\). We observe Aβ\(_{40}\) clustering and dense iron-laden Aβ\(_{42}\) deposition near WM tracts. In the human brain, similar WM loss in the frontal cortex is associated with Aβ and iron accumulation. APP is a common marker for axonal damage and WM shearing\(^{386}\). Myelin loss may make axons more vulnerable to damage. Thus, myelin loss may facilitate APP synthesis at sites of high myelin turnover. Increased APP synthesis may facilitate Aβ cleavage and plaque genesis. Co-injection of large conjugated iron molecule, USPIO-Aβ1-42, with mannitol into a
APP/PS1 mouse\textsuperscript{387} showed increased hypo-intensities in the same areas that we show reduced R\textsubscript{2} and decreased WM in Ch. 2. This suggests that age-induced vulnerabilities to later myelinating mouse brain regions may propagate Aβ deposition.

4.5 Implication for iron induced Aβ\textsubscript{42}/\textsubscript{40} alterations and iron amyloid inclusion

In Ch.3 we observed that Aβ\textsubscript{42} saturation is not altered by brain iron load, however, Aβ\textsubscript{42} plaques appeared denser. In contrast, Aβ\textsubscript{40} plaque load was shown to decrease over time with increasing brain iron accumulation. These findings may indicate an essential role for how iron interacts with plaques, leading to morphological alterations and potential toxicity associated with AD pathology.

The additional C-terminal amino acids of Aβ\textsubscript{42}, valine and isoleucine (or phenylalanine), increase aggregation via a hydrophobic effect. Aβ\textsubscript{40} does not contain these hydrophobic c-terminal ends, thus, it binds more loosely to the Aβ plaque. The ratio between Aβ\textsubscript{42}/\textsubscript{40} may differentiate a primitive/diffuse plaque from a senile cored plaque. Fe\textsuperscript{2+} is found to interact with the N-terminal 1-16 amino acids of Aβ\textsuperscript{54}. Fe\textsuperscript{2+} may interact with this N-terminal end in a similar manner as described with Zn\textsuperscript{2+}, which may retard Aβ\textsubscript{40} fibril formation\textsuperscript{347} and improve Aβ\textsubscript{40} clearance. This would increase Aβ\textsubscript{42}/\textsubscript{40}, creating more cored senile plaques. Many contemporary commercial assays for AD progression utilize Aβ\textsubscript{42}/\textsubscript{40} instead of Aβ\textsubscript{42}/total protein. A higher Aβ\textsubscript{42}/\textsubscript{40} plaque ratio would generate hydrophobic oligomers, which are believed to be more neurotoxic. The cortical and subcortical concentration of iron is higher than that of zinc, so iron may have a more profound effect on Aβ plaque morphology and Aβ clearance. Our data support the finding that Aβ\textsubscript{40} is more easily cleared from the brain than Aβ\textsubscript{42}\textsuperscript{346}. Increased brain iron with exposure to TMHF may highlight how iron alters normal Aβ clearance mechanisms in the brain.
Our observation that the choroid plexus contained higher iron with time and TMHF diet could be indicative of increased CSF iron. An APOE knock-out study illustrates that APOE facilitates iron release from the brain into the CSF\textsuperscript{208}. Increased ferritin in CSF is associated with impaired AD cognitive scores,\textsuperscript{388} as well as predicting prodromal AD\textsuperscript{389}. The inverse may be true as well. Increased choroid plexus iron in our study does not associate with exacerbated Aβ\textsubscript{42} plaque deposition rate or spread, but it may be related to Aβ\textsubscript{40} clearance. Increasing iron levels were found to increase intracellular neuronal and astrocytic APOE while decreasing secreted APOE\textsuperscript{390}. It is hypothesized that Aβ and APOE competitively bind LDLR, which is the proposed Aβ clearance mechanism from the brain. Thus, less APOE during high iron conditions would then allow for better Aβ\textsubscript{40} clearance. This effect would increase Aβ\textsubscript{42}/40 discussed above, propagating the diseased state.

We further show that increased brain iron increases iron-laden amyloid plaques. Increasing iron bound amyloid perturbs storage, clearance, and function of iron. Plaque-bound iron may also help to produce free radicals, increasing the toxicity of Aβ plaques.

4.6 Implication for TMHF induced microglial morphological changes

Microglia are the endogenous macrophages within the brain. These small cells scavenge and metabolize extracellular Aβ and myelin debris, which improves neural function and repair. In basal state, microglia physically prune the iron-rich outer layers of myelin\textsuperscript{391} and prune neuronal synapses to improve function\textsuperscript{392,393}. In this regard, there is overlap between myelination, Aβ deposition, neuronal function, and iron. Age-related myelin degradation and Aβ accumulation burdens the clearance function of microglia during aging\textsuperscript{394}. Surface receptors TREM1 and TREM2 are shown to improve Aβ plaque clearance\textsuperscript{395} and remyelination after cuprizone treatment\textsuperscript{396}. Impairment of both receptors is linked to the AD risk allele rs6910730\textsuperscript{G}
Ferritin is commonly found in activated microglia and phagocytic microglia around Aβ plaques. Microglia may pick up ferritin and labile iron from apoptotic cells, demyelinating fibers or amyloid plaque. Through this process, microglia may serve as an iron and myelin sensor. Microglial pruning of oligodendrocytes, independent of phagocytosis, may be more efficient when their internal iron levels are low. Pruning of the outer myelin bundles may thin WM.

Increased brain iron early in life may improve WM integrity by reducing myelin pruning; this is found in AD patients with H63D hemochromatosis mutation. In addition, microglia have been found to express D1-3 receptors, so they may be further activated with improper DA release or uptake.

We found that TMHF increased brain iron induced microglia dearborization and activation. This effect was noted in both Ch. 2 and 3. Previous research reports the same finding. TMHF appeared to alter microglia morphology toward a phagocytic state during early stages of Aβ42 plaque deposition in the basal ganglia and hippocampus. This effect may be due to increased myelin turnover or ferritin clearance. The early activation of microglia may even facilitate the Aβ40 removal that we observed via a TREM1 moderated lysosomal pathway. However, at 12 months, increased brain iron does not appear to activate microglia more than the normal diet. At this age, mice contain more amyloid and dystrophic microglia, so they may not be able to respond as well to increased brain iron.

4.7 Conclusion

In summary, we propose that metabolic dysfunction, neurotransmitter system dyshomeostasis(eg DA), and impaired re(myelination) with age may predispose the brain to dysfunction and heterogeneous iron accumulation. Increasing brain iron may improve myelin integrity, but iron-induced activation of microglia is associated with oxidative stress and
inflammation that impairs memory function with age. Iron-induced Aβ morphology alterations may further propagate Aβ neurotoxicity and microglial activation.

Treatment strategies that improve WM, enhance microglia maintenance functions, and improve neurotransmitter function, may reduce neural senescence and AD symptoms. Memantine, a drug currently used to treat end-stage AD is believed to function as a glutamatergic antagonist, but there is evidence to suggest that it functions as a D2 receptor agonist. In mouse models of AD, memantine is found to decrease amyloid plaques and improve memory. Enhancing TREM 1 and TREM 2 reduces Aβ plaque load and improves (re)myelination with age.

Our observation that preserved WM volume with TMHF exposure may indicate that oligodendrocyte differentiation and metabolic function is enhanced. However, increasing brain iron leads to oxidative stress and inflammation, which perturb brain function. Acutely administering an iron-containing molecule that enhances oligodendrocyte iron, while at the same time administering a separate compound that chelates extracellular iron, may improve WM integrity and neural plasticity in the aging brain.
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EDUCATION
The Pennsylvania State University, College of Medicine, Hershey, PA
Ph.D. Candidate Neuroscience 2011- Present
University of California, Irvine, Irvine, CA
M.S. Neurobiology of Learning and Memory 2011
Carnegie Mellon University, Pittsburgh, PA
B.S.A. Biological Science and Fine Arts May 2007

AWARDS
Clinical and Translational Science Institute TL1 Predoctoral Career Development Award 2014

PUBLICATIONS
Dietary lipophilic iron regionally increases brain and amyloid iron load in a humanized knock-in APP mutant mouse. Peters DG, Keith KC, Sun D, Yang QX, Pollack A, Haaf MP, Saido TC, Connor JR, Meadowcroft MD. Submitted
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