REGULATION OF IRON MOVEMENT ACROSS THE BLOOD-BRAIN BARRIER

A Dissertation in
Cellular and Developmental Biology
by
Kari Ann Duck

© 2016 Kari Ann Duck

Submitted in Partial Fulfillment
of the Requirements
for the Degree of
Doctor of Philosophy

December 2016
The dissertation of Kari Ann Duck was reviewed and approved* by the following:

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

Colin J. Barnstable  
Professor and Chair of the Department of Neural and Behavioral Sciences

Patricia J. McLaughlin  
Professor of the Department of Neural and Behavioral Sciences

Ian A. Simpson  
Professor of the Department of Neural and Behavioral Sciences

Zhi-Chun Lai  
Professor of Biology, Biochemistry and Molecular Biology  
Head of the Intercollege Graduate Degree Program in Cell and Developmental Biology

*Signatures are on file in the Graduate School
ABSTRACT

Iron is one of the most important micronutrients due to its involvement as a cofactor in various essential processes in the body such as oxygen transport and the electron transport chain. For neuroscientists, iron is of critical interest because of its function in both neurotransmitter synthesis and myelination. The study of iron in the brain has revealed both gradual accumulation as we age and also dyshomeostasis in disease. Regulation of iron movement into the brain is managed by a blood-brain barrier (BBB). The BBB is comprised of the microvasculature throughout the brain and is characterized by tight junctions that allow for regulation of nutrient transport between the blood and the brain. Despite the importance of iron in brain function, research on the mechanism by which iron enters the brain is limited. To date, there are two potential mechanisms that have been proposed: (i) transcytosis of the iron transporter, transferrin (Tf) and (ii) endocytosis and release into the cell through divalent metal transporter 1 (DMT-1). Our research efforts have revealed both mechanisms are present. Two mechanisms may be required because the direct transcytosis mechanism overlooks the iron needs of the endothelium and the second mechanism identifies a pathway by which the endothelium and the brain iron needs are met.

The overall hypothesis for this thesis is that iron movement into the brain is regulated by the brain. Both cell culture and animal models were utilized to examine the two key questions regarding brain iron uptake that were investigated: (i) what are the mechanisms of iron movement into the brain and (ii) how is iron movement into the brain regulated. In the first subset of cell culture studies, we demonstrated increased release of iron from the endothelium when exposed to apo-Tf (iron poor), however hepcidin, a protein known to block iron export by binding to the iron export protein ferroportin, had no impact on iron release alone or on iron release induced by apo-Tf. These data identify iron poor transferrin in extracellular fluid in the brain as a potential
mechanism to stimulate iron release from the BBB. The second set of studies focused on the impact of both apo-Tf and DMT-1 on transport of iron from the blood side of the cultured model to the brain side. A DMT-1 inhibitor, XEN602, was implemented to investigate the role of DMT-1 in iron transport. Neither apo-Tf nor XEN602 had a significant effect on total transport of 59Fe or Tf. Of note, however, is that XEN602 exposure did not result in increases in iron within the cells, which suggests that a majority of the iron seen in the brain-side chamber was transcytosed when the endocytic model was blocked.

Given, that the first set of studies demonstrated regulation of iron release and transport in the BBB model, we next sought to evaluate how a neurological disorder that was associated with brain iron deficiency would impact iron transport and release. Specifically, we obtained cerebrospinal fluid (CSF) from patients with restless legs syndrome (RLS) to test the hypothesis that the brain iron deficiency in RLS results in increased signaling to upregulate brain iron uptake. First, we found that CSF from both control and RLS patients who had low hemoglobin relative to controls had decreased transport of iron across the BBB model. The low hemoglobin RLS group was also associated with higher iron transport than the low hemoglobin non-RLS control group. Moreover, iron release had a modest but statistically significant positive correlation to systemic hemoglobin levels, but there was no correlation of transport to either hemoglobin or serum ferritin. The latter results are important because serum ferritin levels are used clinically to identify RLS patients as candidates for intravenous iron therapy.

The data from the cell culture models strongly suggest, consistent with our hypothesis, that there are signals on the brain side (e.g. CSF) of the BBB that regulated iron movement across or from the BBB. Therefore, we examined 3-month old mice carrying an H67D mutation in the HFE gene. This mutation promotes excess iron accumulation in most organs of the body and in its extreme case is associated with hemochromatosis. We found that the H67D/H67D mice had elevated total brain iron levels, but took up comparable amounts of iron into the brain to the
wildtype mice over 24-hour and 5-day time periods. This finding supports the hypothesis that brain iron transport is regulated by the brain even in the presence of a mutation that promotes iron uptake. We also observed significantly more 59Fe uptake into the female brain when compared to the male brain irrespective of genotype. Notably, this study is unique in that it considers the microvasculature as an independent compartment of the brain. This investigation identified iron uptake and retention within the microvessels over a 5-day time period, substantiating the hypothesis that the BBB can function as an iron reservoir for the brain.

Together, the data presented in this thesis confirm that the BBB functions as a central regulatory hub for brain iron uptake. We have demonstrated that there is regulation of release of iron that comes from the brain side of the BBB. The data with the mutant mice suggest that once the brain growth has plateaued, brain iron uptake is constant even if there is a mutation present that promotes uptake. There is also a significant sex effect for brain iron uptake. The data in this thesis provide new insights into regulation of iron uptake into the brain and, because the signals for brain iron status come from the brain, the data call into question the use of iron supplementation strategies to improve cognitive impairment beyond the developmental stage.
### TABLE OF CONTENTS

LIST OF FIGURES .................................................................................................................. viii

LIST OF TABLES .................................................................................................................... xii

LIST OF ABBREVIATIONS ...................................................................................................... xiii

ACKNOWLEDGEMENTS ........................................................................................................ xv

Chapter 1 Iron uptake and transport across physiological barriers ........................................ 1

1.1. Abstract ............................................................................................................................ 1
1.2. Introduction ...................................................................................................................... 1
1.3. Iron Transport in the Duodenum ....................................................................................... 3
   1.3.1. Mechanism of Iron Transport in the Duodenum ......................................................... 5
   1.3.2. Regulation of Iron Transport Across the Intestinal Lumen ................................. 6
1.4. Iron Transport Across the Placenta ................................................................................. 8
   1.4.1. Mechanism of Iron Transport at the Placenta ......................................................... 9
   1.4.2. Regulation of Iron Transport Across the Placenta ............................................... 12
1.5. Iron Transport Across the BBB ..................................................................................... 15
   1.5.1. Mechanism of Iron Uptake at the BBB ................................................................. 17
   1.5.2. Regulation of Iron Transport at the BBB ............................................................... 22
1.6. Importance of the HFE Protein in Barrier Iron Transport ........................................... 24
1.7. Summary of the Similarities of Gut and Placenta Iron Uptake Mechanisms to BBB Iron Uptake Mechanisms ................................................................. 27
1.8. References ..................................................................................................................... 30

Chapter 2 The Role of the Blood-Brain Barrier in Maintaining Brain Iron Status ................. 41

2.1. Abstract ............................................................................................................................ 41
2.2. Introduction ...................................................................................................................... 42
2.3. Materials and Methods ................................................................................................. 43
   2.3.1. Radiolabeling of Transferrin ............................................................................... 43
   2.3.2. Cell Culture ........................................................................................................... 44
   2.3.3. Release Assay ....................................................................................................... 44
   2.3.4. Transport Assay .................................................................................................... 45
   2.3.5. Protein Expression Assay .................................................................................... 46
   2.3.6. Gene Expression Assay ....................................................................................... 46
   2.3.7. Statistical Analyses ............................................................................................... 47
2.4. Results ............................................................................................................................. 48
   2.4.1. Regulation of Iron Release in an In Vitro BBB Model ........................................... 48
   2.4.2. Mechanisms and Regulation of Iron Transport in an In Vitro BBB Model ........ 53
2.5. Discussion ....................................................................................................................... 58
2.6. References ..................................................................................................................... 63

Chapter 3 The role of sex and a common HFE gene variant in brain iron uptake ................. 66
Chapter 4 Evaluation of Brain Iron Uptake using Cerebrospinal Fluid from Restless Legs Syndrome patients

4.1. Abstract ............................................................................. 89
4.2. Introduction ....................................................................... 89
4.3. Materials and Methods .......................................................... 91
   4.3.1. Radiolabeling of Transferrin .............................................. 91
   4.3.2. Cell Culture ................................................................... 91
   4.3.3. Release Assay ................................................................. 92
   4.3.4. Transport Assay ............................................................... 93
   4.3.5. Statistical Analyses .......................................................... 94
4.4. Results .............................................................................. 94
4.5. Discussion ......................................................................... 98
4.6. References ......................................................................... 101

Chapter 5 Regulation of Brain Iron Status – Summary and Implications ........................................ 104

5.1. Summary of Main Findings of Dissertation .................................. 104
5.2. When Are There Signals to the BBB About Brain Iron Status? .......... 105
5.3. Are There Conditions Where Iron Supplementation Works? .............. 110
5.4. Is the Nigrostriatal System the Only Area Where Iron Status is Alterable? 112
5.5. Diseases with Too Much Iron – Is There Anything That Can Be Done? .... 115
5.6. How does sex affect brain iron uptake? ..................................... 118
5.7. Conclusions ...................................................................... 119
5.8. References ...................................................................... 121
LIST OF FIGURES

Figure 1-1: Schematic of intestinal iron transport Briefly, the primary mechanism by which iron is taken up by the enterocyte is through DMT-1 on the luminal membrane after reduction by DcytB. Iron has also been suggested to be transported as heme through HCP1 and as ferritin, but these mechanisms are controversial (heme) or less investigated (ferritin). Once in the intracellular labile iron pool, the iron can be stored in ferritin or exported into the body circulation through ferroportin. The ferroxidase, hephaestin, converts the ferrous iron that is released to ferric iron for use by transferrin. ...................................................... 4

Figure 1-2: Schematic of placental iron transport In brief, placental iron transport is completely transferrin-dependent. Transferrin binds to transferrin receptor on the maternal membrane and is endocytosed. The pH decrease in the endosome causes iron to dissociate from transferrin after which it can be transported into the cytoplasm through DMT-1. Once in the cytoplasm, iron can be stored in ferritin, associate with heme, be incorporated into the labile iron pool, or be transported to the fetal circulation through ferroportin. The ferroxidase on the placenta is called zyklopen and functions to convert the ferrous iron released by ferroportin to ferric iron, which is then usable by transferrin. ................................................................. 11

Figure 1-3: Schematic of brain iron uptake The process by which iron crosses the BBB has recently been modeled (Simpson et al. 2015). Route 1 represents the currently accepted paradigm of the endothelial cell as a passive conduit in which transferrin binds to its receptor on the luminal membrane, traverses the cell and is deposited into the brain. Route 2 represents the more realistic and data-based model in which transferrin is endocytosed after binding to transferrin receptor. The iron is then released by transferrin within the endosome and transported into the cytoplasm through DMT-1. The intracellular iron can be stored in ferritin or it can be released into the brain through ferroportin. This model accounts for the iron needs of the endothelial cells. Route 3 depicts a potential mechanism by which ferritin can transport iron across the BBB. The possibility of this mechanism has been demonstrated, but further study is required to better understand it. ................................. 19

Figure 1-4: Similarities and differences in iron transport at physiological barriers When comparing iron transport at the gut, the placenta, and the BBB, there are three key similarities seen at all barriers. First, each barrier appears to demonstrate presently unidentified alternative transport mechanisms. Secondly, DMT-1 is involved in iron trafficking at each barrier. Finally, all three barriers also utilize ferroportin on their basolateral membrane for iron export. Evaluation of each overall mechanism suggests that the BBB is most similar to the placenta. Iron transport at both barriers is primarily transferrin-dependent and involves a mechanism by which iron is released from transferrin in the endosome for export into the intracellular iron pool. Unlike either of the other barriers, the gut requires uptake of non-transferrin bound iron. The BBB appears to be unique in its ability to transcytose transferrin-
bound iron. Additionally, the potential for heme transport has been hypothesized in both the placenta and the gut, but there is no evidence to suggest that this occurs at the BBB. In terms of regulation, the BBB demonstrates transferrin-transferrin receptor feedback signaling that is similar to what is observed in the crypt cells of the gut. Meanwhile, the placental regulatory system is unique in that iron transport is under the control of both the mother and the fetus. ................................................................. 28

Figure 2-1: Iron release from BBMVECs in a modeled BBB after exposure to 10 uM apo-Tf, 100 uM DFO, and 500 nM hepcidin. A) 500 nM hepcidin treatment had no significant effect on $^{59}$Fe release from BBMVEC. B) 10 uM apo-Tf significantly increased release into the basal chamber from BBMVEC. The apo-Tf induced iron release was not altered by 500 nM hepcidin treatment. C) Apo-Tf, a physiologically-relevant iron chelator, and DFO, a pharmacologic iron chelator, cause significant release of iron into the basal chamber over 4 hours. Concentrations were selected based on documented standard in vitro conditions. Of note, 100 uM DFO can bind 5 times more iron than 10 uM apo-Tf. Data are represented as mean ± SEM, n = 3 per condition. *p < 0.05, ***p < 0.001, ****p<0.0001. ................................. 49

Figure 2-2: Effect of treatment with Fe-NTA, apo-Tf, and hepcidin on ferroportin protein expression in BBMVECs. A) Representative western blot of ferroportin and β-actin. B) There was a consistent decrease of 38% in ferroportin when BBMVECs were treated with Fe-NTA and this decrease was not affected by exposure to 500nM hepcidin. The decrease in ferroportin in response to apo-Tf was eliminated when the cells were treated with a combination of Fe-NTA, apo-Tf and hepcidin. Ferroportin was expressed as a ratio of the protein to β-actin in the endothelial cells. The ratio was normalized to a common liver sample to account for intra-experimental variance. Data are represented as mean ± SEM, n = 3 per condition. *p < 0.05. ................................................................. 50

Figure 2-3: mRNA expression in BBMVEC after treatment with Fe-NTA, apo-Tf, and hepcidin. A) There was an overall trending increase in ferroportin mRNA expression in BBMVECs. Hepcidin alone caused a 58% increase and iron loading caused an 86% increase in ferroportin mRNA. B) There was a trending decrease in transferrin receptor expression. Specifically, hepcidin in the presence of iron resulted in a 36% decrease in transferrin receptor mRNA levels. C) L-ferritin mRNA was not affected by release treatments. D) H-ferritin mRNA levels remained consistent in the presence of the release condition treatments. Data are represented as mean ± SEM, n = 3 per condition................................................................. 52

Figure 2-4: Effect of a DMT-1 inhibitor, XEN602, on iron transport across the BBB. A) Total $^{59}$Fe transported to the basal chamber was not significantly reduced by exposure to the XEN602. B) The total lysate-associated $^{59}$Fe was not altered by XEN602 treatment. C) Transferrin flux into the basal chamber was reduced 56% by XEN602 treatment. D) The ratio of bound $^{59}$Fe to free $^{59}$Fe was reduced when exposed to XEN602. E) XEN602 caused a significant decrease in protein-bound $^{59}$Fe transported into the basal chamber. F) Free $^{59}$Fe transport into the basal chamber was reduced in response to XEN602 treatment. ................................................................. 54
Figure 2-5: Effect of apo-Tf treatment on iron transport in an in vitro BBB model.
A) No significant changes were observed in total iron accumulation in the basal chamber. B) Lysate-associated iron was not altered by treatment with apo-Tf with or without XEN602. C) Transferrin flux was not altered by the apo-Tf or the apo-Tf with XEN602 in the basal chamber. D) Total protein-bound iron was not affected by the treatment with apo-Tf or apo-Tf with XEN602. E) The total free iron was reduced when apo-Tf was present in the basal chamber and this was not altered by XEN602. F) The ratio of bound-to-unbound iron was increased in the basal chamber for the apo-Tf treatment conditions. Data are represented as mean ± SEM, n = 3 per condition. *p < 0.05. ........................................................................................................ 56

Figure 2-6: mRNA expression in BBMVEC after treatment with apo-Tf and XEN602 alone or apo-Tf and XEN602 combined. A) Ferroportin was decreased by 20% with apo-Tf treatment. B) There was a 29% decrease in transferrin receptor mRNA expression after BBMVEC exposure to apo-Tf and a 15% decrease with XEN602. C) L-Ferritin mRNA was, on average, decreased by 23%. D) H-Ferritin mRNA was not affected by transport study treatments. Data are represented as mean ± SEM, n = 3 per condition......................................................... 57

Figure 2-7: Schematic representation of DMT-1 inhibitor effect on iron transport. XEN602, as expected, caused a decrease in the amount of free iron being released into the basal chamber. We also saw that protein-bound iron transport was reduced with the use of XEN602. The presence of the DMT-1 inhibitor forces recycling to the membrane of origin for the vesicle whereas in a normal setting, after release of ferrous iron from the compartment of uncoupling of receptor and ligand (CURL) via DMT-1, the vesicle could recycle to either membrane......................................................... 59

Figure 3-1: Total brain iron. Total brain iron was measured in H67D/H67D and wild type mice by atomic absorption. There was significantly more iron in the brain of H67D/H67D mice when compared to their wild type counterparts. Iron in H67D/H67D males was ~37% higher than wild type males while iron in H67D/H67D females was ~61% higher than wild type females. n = 5/group. ***p < 0.001, ****p < 0.0001. ................................................................. 73

Figure 3-2: 59Fe accumulation in the livers of wild type and H67D/H67D mice. A) At 24 hours after injection, livers were collected, weighed, and radioactivity was measured. H67D/H67D females exhibited significantly more 59Fe retention in the liver, but no other changes were observed. B) At 5 days after injection, there were no significant differences in 59Fe liver accumulation across genotypes, but there was significantly more 59Fe retained in the liver of female mice when compared to their male counterparts. *p < 0.05........................................................................................................ 74

Figure 3-3: 59Fe accumulation in whole brain. A) At 24 hours after injection with 59Fe-Tf, brains were homogenized and radioactivity was measured. No significant sex or genotype effects were observed. B) At 5 days after injection with 59Fe-Tf, brains were homogenized and radioactivity was measured. There was significantly more 59Fe present in the brains of female mice than in the brains of male mice for both genotypes studied. C) Changes in 59Fe between the 24-hour and 5-day time points
was calculated. Wild type females exhibited significantly less change than the wild type males, which had reduced $^{59}\text{Fe}$. *p < 0.05.

**Figure 3-4:** $^{59}\text{Fe}$ accumulation in an isolated cortical brain fraction. A) After 24 hours, no significant sex or genotype effects were observed. B) Five days after injection, females for both genotypes exhibited significantly more cortical $^{59}\text{Fe}$ accumulation when compared to males within the same genotype. C) There were no significant differences in the change seen in cortical brain levels of $^{59}\text{Fe}$. *p < 0.05.

**Figure 3-5:** $^{59}\text{Fe}$ accumulation in a myelin-containing brain fraction. A) No significant sex or genotype effects were seen at 24 hours after injection. B) At 5 days after injection, however, there was significantly more $^{59}\text{Fe}$ in the myelin-containing brain fraction of females when compared to males within the same genotype. C) The change in iron associated with the myelin-containing fraction was calculated. The wild type males exhibited a greater reduction in $^{59}\text{Fe}$ over the 5-day time period than wild type females or H67D/H67D males. *p < 0.05.

**Figure 3-6:** $^{59}\text{Fe}$ accumulation in the brain microvasculature. A) Within 24 hours after injection, the brain microvasculature accumulated measurable levels of $^{59}\text{Fe}$. B) After 5 days, the brain microvasculature retained $^{59}\text{Fe}$. C) The microvasculature exhibited almost no change in $^{59}\text{Fe}$ over the 5-day time course studied here, indicating its ability to retain iron and function as an iron reservoir.

**Figure 4-1:** Effect of CSF from healthy and RLS patients on iron transport and release in an in vitro BBB model. A) Iron flux across the in vitro BBB model was not significantly different between the control and RLS groups. B) CSF from RLS patients had no significant impact on iron release from the cultured BBB model. *p < 0.05.

**Figure 4-2:** Correlation of iron transport and release to systemic iron measures. A) Transport exhibited a weak positive correlation to systemic Hgb levels (R-square = 0.33, p = 0.004). B) There was a positive correlation between systemic Hgb levels and iron released from the in vitro BBB model (R-square = 0.63, p = 0.05). C) There was no significant correlation between iron transport and serum ferritin (R-square = 0.05, p = 0.35). Grey squares represent control patients and black circles represent RLS patients.
LIST OF TABLES

Table 1-1: Brain disorders associated with brain iron accumulation or brain iron deficiency.............................................................................................................................................. 17

Table 2-1: TaqMan qPCR Probes. .......................................................................................................................................................................................... 47

Table 4-1: Patient demographics for RLS CSF in vitro BBB assays................................................................. 93
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-syn</td>
<td>α-Synuclein</td>
</tr>
<tr>
<td>AA</td>
<td>Atomic Absorption</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>Apo-Tf</td>
<td>Iron-poor Transferrin</td>
</tr>
<tr>
<td>β(_2)M</td>
<td>β(_2) Microglobulin</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-Brain Barrier</td>
</tr>
<tr>
<td>BBMVEC</td>
<td>Bovine Brain Microvascular Endothelial Cell</td>
</tr>
<tr>
<td>BRB</td>
<td>Blood-Retina Barrier</td>
</tr>
<tr>
<td>BREC</td>
<td>Bovine Retinal Endothelial Cell</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts Per Minute</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
</tr>
<tr>
<td>DcytB</td>
<td>Duodenal Cytochrome B</td>
</tr>
<tr>
<td>DFO</td>
<td>Deferoxamine</td>
</tr>
<tr>
<td>DFP</td>
<td>Deferiprone</td>
</tr>
<tr>
<td>DMT-1</td>
<td>Divalent Metal Transporter 1</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
</tr>
<tr>
<td>HCP1</td>
<td>Heme Carrier Protein 1</td>
</tr>
<tr>
<td>Hgb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>HH</td>
<td>Hereditary Hemochromatosis</td>
</tr>
<tr>
<td>HO</td>
<td>Heme Oxygenase</td>
</tr>
<tr>
<td>HPX</td>
<td>Hypotransferrinemic</td>
</tr>
<tr>
<td>IRP</td>
<td>Iron Regulatory Protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin Basic Protein</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>NBIA</td>
<td>Neurodegeneration with Brain Iron Accumulation</td>
</tr>
<tr>
<td>NTA</td>
<td>Nitrilotriacetic Acid</td>
</tr>
<tr>
<td>NTBI</td>
<td>Non-Transferrin Bound Iron</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s Disease</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Real-Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation Assay</td>
</tr>
<tr>
<td>RITC</td>
<td>Rhodamine B Isothiocyanate</td>
</tr>
<tr>
<td>RLS</td>
<td>Restless Legs Syndrome</td>
</tr>
<tr>
<td>Tf</td>
<td>Transferrin</td>
</tr>
<tr>
<td>TBI</td>
<td>Transferrin-bound Iron</td>
</tr>
<tr>
<td>TfR</td>
<td>Transferrin Receptor</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to dedicate this thesis to the three people who have devoted themselves to helping me reach my every goal – my family. I will always be most grateful to my family for seeing the light when I wasn’t sure if there was anything left but darkness. To my father – thank you for always being proud of me and for always providing humor in tough moments. To my sister – thank you for always loving your baby girl. You have provided such valuable life insights and encouragements without which I would not have reached this point. To my mother – thank you for always being just a phone call away. Your unwavering commitment to keeping me strong and sound as I moved through not only this graduate program but also through life struggles has made me the woman I am today.

I would like to also dedicate this thesis to the memory of my late grandmother, Henrietta Duck. Your regular phone calls and cards for every holiday brought small moments of joy to my life and reminded me that I am loved and supported by those at home. My only regret is that you are not here to see me finally complete this body of work.

To my darling Alexander, sometimes life sends us love when we least expect it and most need it. You have been the rock keeping me steady in these last few months and I am so thankful for your patience and understanding. I will forever be amazed by your ability to perceive my needs and meet them without my saying a word.

To my dissertation advisor, Dr. Connor, I do not think that words can express my level of thanks. You provided me with a laboratory home when I could not find one. Your support and understanding throughout the last years has been crucial to my development as a young scientist. My committee has also proven a valuable source of guidance throughout my graduate career. For this, I give you all my sincerest thanks.
I wish to thank all of the members of the Connor Laboratory for their contributions to my success here. To Becky – thank you for being there day in and day out to hear my struggles and offer your support. To Beth – you were essential to getting through the radioactive animal studies and for that, I will always be most grateful. I also would like to say thank you to Dominique, Mandy, Stephanie, and Wint who were vital in supporting me both professionally and personally. Finally, I would like to give thanks to a special group of people – my “blondetourage”. Anne, you have stood by me in and out of the lab for the last several years and I would not have survived without you. Oliver, your addition to the group brought a fresh sense of humor and unique support that made the toughest of my years here a happier time.

Finally, to all of the friends that I have made along the way, I thank you all. From my friend Laura, whom I met on the first day of classes, to my crafting girls of the last 1.5 years, you have all been instrumental in keeping me sane outside of the laboratory. For this, I will be forever grateful.
Chapter 1
Iron uptake and transport across physiological barriers

1.1. Abstract

Iron is an essential element for human development. It is a major requirement for cellular processes such as oxygen transport, energy metabolism, neurotransmitter synthesis, and myelin synthesis. Despite its crucial role in these processes, iron in the ferric form can also produce toxic reactive oxygen species. The duality of iron’s function highlights the importance of maintaining a strict balance of iron levels in the body. As a result, organisms have developed elegant mechanisms of iron uptake, transport, and storage. This review will focus on the mechanisms that have evolved at physiological barriers, such as the intestine, the placenta, and the blood-brain barrier (BBB), where iron must be transported. Much has been written about the processes for iron transport across the intestine and the placenta, but less is known about iron transport mechanisms at the BBB. In this review, we compare the established pathways at the intestine and the placenta as well as describe what is currently known about iron transport at the BBB and how brain iron uptake correlates with processes at these other physiological barriers.

1.2. Introduction

Iron is an essential, multifunctional micronutrient that has been shown to function in oxygen transport through its role in hemoglobin, as a crucial cofactor in the electron transport chain, and as a cofactor during DNA synthesis (Beard et al. 1996). The multifunctionality of iron stems from its ability to easily transition between its two oxidation states, ferrous (Fe^{2+}) and ferric (Fe^{3+}).
The process by which iron transitions between states is known as Fenton chemistry represented as:

\[
\begin{align*}
(1) \quad \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{HO}^\cdot + \text{OH}^- \\
(2) \quad \text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{2+} + \text{HOO}^\cdot + \text{H}^+
\end{align*}
\]

During this reaction, ferrous iron reacts with hydrogen peroxide to generate a hydroxyl radical and ferric iron reacts with hydrogen peroxide to form a hydroperoxyl radical (reviewed in Chevion, 1988; Stohs and Bagchi, 1995) (Chevion 1988; Stohs and Bagchi 1995).

Iron imbalance, whether too much or too little, can be harmful. Too little iron can cause iron deficiency with subsequent anemia, which is the most common nutrient disorder worldwide (Iron Deficiency Anemia 2016). The condition presents with decreased red blood cell production and reduced hemoglobin levels and is associated with fatigue and weakness in patients. While having too little iron can have negative effects, too much iron can also be harmful. When iron participates in Fenton chemistry, it generates toxic free radicals and reactive oxygen species, which can be detrimental to cell health leading to damage of lipids, proteins, and DNA, ultimately resulting in cell death. Thus, it is crucial for iron levels to be tightly regulated. Physiological levels of iron typically remain constant due to a minimal excretion mechanism so homeostasis is maintained by strict regulation of iron uptake at the gut. During pregnancy, it is important to maintain iron homeostasis in not only the mother, but also in the fetus. The placenta acts as a barrier at which iron transfer can be regulated by both the mother and the fetus. Similarly, the brain also must be protected from iron imbalances because its high rate of oxygen consumption results in high iron requirements yet its concentration of lipids makes it particularly vulnerable to oxidative damage (Raichle and Gusnard 2002; Sokoloff et al. 1955). To access the brain, iron
must be transported across the blood-brain barrier (BBB). This barrier is composed of the brain microvasculature, which is distinct from other microvessels in the body because it forms tight junctions, blocking passive diffusion between cells into the brain. While iron transfer at both the gut and the placenta have been studied for decades, research into the mechanisms and regulation at the BBB are less understood. In this review, we compare and contrast mechanisms and regulation of iron transport at the gut, placenta, and BBB as an attempt to further elucidate important factors to be studied in the BBB model.

1.3. Iron Transport in the Duodenum

The gut functions as the key modulator of iron concentration in the body. A sophisticated mechanism of intestinal uptake and regulation has evolved so that sufficient quantities of iron are absorbed daily to fulfill daily requirements and maintain sufficient iron stores while not allowing for excessive amounts of iron to accumulate (Figure 1-1). The importance of the gut’s role in maintaining iron homeostasis through absorption stems from the body’s lack of an iron excretion mechanism. Skin exfoliation, sloughing of intestinal epithelium, and menstruation remain the three primary processes by which iron is excreted, as very little is removed from the body in urine or feces (Cole et al. 1971; Finch et al. 1970; Green et al. 1968). On average, the body loses only 1-2 mg of iron/day (Cole et al. 1971; Finch et al. 1970; Green et al. 1968). At the same time, the body uses 20-25 mg of iron/day, most of which is required for erythropoiesis (Bothwell et al. 1958a; Hentze et al. 2004). The majority of the body’s iron is stored to address both acute and chronic iron needs, making regulation of absorption at the gut crucial so that the body’s iron needs are met without depleting stores. Discussed in more detail below, the enterocytes of the intestines are renewed regularly from a population of crypt cells that are exposed to systemic iron
and alter their iron management protein profiles accordingly to alter the amount of iron transport they will support.

**Figure 1-1**

Briefly, the primary mechanism by which iron is taken up by the enterocyte is through DMT-1 on the luminal membrane after reduction by DcytB. Iron has also been suggested to be transported as heme through HCP1 and as ferritin, but these mechanisms are controversial (heme) or less investigated (ferritin). Once in the intracellular labile iron pool, the iron can be stored in ferritin or exported into the body circulation through ferroportin. The ferroxidase, hephaestin, converts the ferrous iron that is released to ferric iron for use by transferrin.
1.3.1. Mechanism of Iron Transport in the Duodenum

The mechanism by which iron moves from the intestinal lumen into the bloodstream occurs in three stages – 1) Luminal uptake and transport across the apical membrane, 2) Transfer to the basolateral membrane, and 3) Transport across the basolateral membrane into the circulation. In the first stage of transport, iron must cross from the lumen of the intestine and into the enterocyte. The first mechanism by which iron gets into the enterocyte requires that ferric iron be reduced by duodenal cytochrome B (DcytB) to its ferrous form (Luo et al. 2014; McKie et al. 2001; Vlachodimitropoulou et al. 2010). The ferrous iron can then be transported through divalent metal transporter 1 (DMT-1), a known mediator of cationic metal transport that is present at the apical membrane of the enterocyte (Canonne-Hergaux et al. 1999; Gunshin et al. 1997; Mackenzie and Garrick 2005). Heme iron is imported by the enterocyte, but the mechanism remains undetermined. Heme carrier protein 1 (HCP1) was identified in the enterocyte and later shown to function as a low-affinity heme transporter into the enterocyte (Le Blanc et al. 2012; Shayeghi et al. 2005). HCP1’s role as the primary mode of heme uptake remains in question, however, as research has implicated this protein in folate transport rather than in heme transport (Qiu et al. 2006). Any heme that may be taken up by the cell is catabolized by heme oxygenase (HO), causing the release of ferrous iron into the cell’s labile iron pool (Raffin et al. 1974; West and Oates 2008). Another potential direct source of iron is ferritin which can traverse the enterocyte and accumulate in the blood, but the pathway by which this occurs remains unstudied (Williams and Hemmings 1978).

Upon entering the enterocyte, iron can be transported to and then across the basolateral membrane or it can be taken up by ferritin, an iron storage protein with ferroxidase activity. Ferroportin, an iron transport protein, has been observed on the basolateral membrane of the
enterocyte (Donovan et al. 2005). Once at the basolateral membrane, iron passes through ferroportin in its ferrous form. Ferroportin is coupled with hephaestin, a ferroxidase present on the basolateral membrane (Han and Kim 2007; Yeh et al. 2009; Yeh et al. 2011). Hephaestin functions to convert ferrous iron to ferric iron which can then be taken up by apo-Tf in the serum to be transported throughout the body (Chen et al. 2004).

1.3.2. Regulation of Iron Transport Across the Intestinal Lumen

The gut has developed an intricate system of checks and balances to regulate iron transport across the enterocyte. The enterocytes of the absorptive intestinal epithelium are the product of crypt cell maturation. Crypt cells have access to the body’s circulation allowing them to take up iron from the blood. When $^{59}$Fe was injected intravenously, acute accumulation was evident in crypt cells, but not enterocytes (Bedard et al. 1976). The amount of iron accumulation in the crypt cell was dependent on systemic iron status (Bedard et al. 1976). Thus, it was proposed that crypt cells function as a sensor of systemic iron status that can alter their levels of iron management proteins, lead to changes in the iron transport when the crypt cells mature into enterocytes. Later studies demonstrated that crypt cells expressed both transferrin and transferrin receptors that can take up iron from the circulation (Anderson et al. 1994; Levine and Woods 1990). Further characterization of the crypt cells demonstrated ferritin mRNA, but minimal ferritin protein while the mature cells expressed ferritin protein at levels proportional to systemic iron levels (Oates and Morgan 1997). That ferritin mRNA in the crypt cell is not translated until the cell matures provides additional support for the hypothesis that crypt cells can sense systemic iron and alter enterocyte protein expression profile upon maturation into an enterocyte.
The role of the ionic iron transporter, DMT-1, became of interest because studies had shown that crypt cells take up transferrin bound iron from blood while the majority of iron taken up from the intestine by mature enterocytes was non-transferrin bound (Waheed et al. 1999). It was shown that in both iron deficiency and in hereditary hemochromatosis, a disease primarily characterized by elevated iron due to a mutation in the HFE protein, both result in iron deficient crypt cells with elevated levels of DMT-1 mRNA (Fleming et al. 1999b). The Belgrade rat, which expresses a mutated DMT-1 protein has been used to understand the role of DMT-1 in regulating intestinal iron absorption. These rats have decreased levels of iron and increased DMT-1 gene expression in the enterocyte, as is also seen in the case of iron deficiency where iron absorption across the enterocyte is increased (Knöpfel et al. 2005; Oates et al. 2000). This increase in DMT-1 gene expression indicates that the system is still responsive to the iron deficiency caused by the DMT-1 mutation but that the mutation keeps the Belgrade animal from increasing its iron absorption (Bowen and Morgan 1987; Edwards et al. 1980; Farcich and Morgan 1992; Garrick et al. 1993).

Intestinal iron transport into the body is also regulated at the basolateral membrane of the enterocyte. Here, the enterocyte expresses an iron transporter known as ferroportin (Donovan et al. 2005). Ferroportin is regulated by hepcidin, a peptide that binds to ferroportin and induces internalization of the exporter (Nemeth et al. 2004). Ferroportin is then degraded by the proteasome, reducing iron export. While systemic iron levels can affect uptake into the enterocyte via DMT-1 modulation starting with the crypt cells, they can also affect uptake by adjusting synthesis of hepcidin peptide. When the body has adequate or too much iron, the liver secretes hepcidin, which can then act on the enterocyte to decrease its ferroportin expression and to reduce iron release into the bloodstream. The decrease in iron absorption allows the body to gradually return to normal iron levels and can then be adjusted to maintain normal systemic iron levels. The converse is true for iron deficiency. In this case, the body has relatively low levels of circulating
hepcidin allowing ferroportin expression to remain at the basolateral membrane. The enterocytes can continue to release iron until normal systemic iron levels are obtained. As the body attains sufficient amounts of iron, the liver will gradually adjust hepcidin synthesis to maintain normal levels while preventing iron overload.

More recently, H-Ferritin has been implicated in the regulation of iron uptake. Conditional knockdown of H-Ferritin in enterocytes led to a two-fold increase in iron absorption despite iron-overload induced decreases in DMT-1 and DcytB mRNA levels (Vanoica et al. 2010). These studies become significant because it implies that the enterocyte is not properly responding to iron absorption cues. The animals exhibited elevated liver iron and, subsequently, elevated hepcidin levels, but enterocyte ferroportin protein was elevated despite no changes in ferroportin mRNA (Vanoica et al. 2010). Thus, the loss of enterocyte H-Ferritin must override not only the contribution of crypt cells to iron regulation, but also alters the response of ferroportin to hepcidin. These studies demonstrate that intracellular communication is more important in the hierarchy of intestinal iron absorption than current models propose.

1.4. Iron Transport Across the Placenta

The placenta serves as the interface between mother and fetus. It regulates nutrient transport to the fetus including the transport of iron. Of note, iron transfer at the placenta is uni-directional. Once iron is taken up by the placenta, it cannot re-enter the maternal circulation (Srai et al. 2002), emphasizing the hierarchy of placental iron transfer. Evidence shows that maternal iron stores will be depleted before those of the fetus (Gambling et al. 2009). It is also known that the rate of iron transfer across the placenta increases over the course of pregnancy revealing the importance of iron in developmental processes (Davies et al. 1959; Glasser et al. 1968; McArdle et al.
This increase occurs at a rate not explainable simply by increased transferrin receptors as a result of a larger surface area from placental growth (Jones and Fox 1991). Thus, the changing fetal iron requirements throughout pregnancy suggest the need for a changing placental iron transport mechanics to account for increases in iron transport.

1.4.1. Mechanism of Iron Transport at the Placenta

The process of iron transport at the placenta is unlike the mechanism seen in the gut (Figure 1-2). Unlike transport the gut, uptake of iron into the placenta is exclusively transferrin-mediated. The crucial role of Tf in placental iron uptake is evidenced by the hypotransferrinemic (hpx) mouse that has a severe Tf deficiency (Bernstein 1987; Huggenvik et al. 1989; Trenor et al. 2000). In these mice, neonates are severely anemic and survive to only 2 weeks of age unless treated with serum or Tf (Bernstein 1987; Craven et al. 1987; Dickinson and Connor 1994; Huggenvik et al. 1989; Simpson et al. 1993; Trenor et al. 2000). While the source of iron in the fetus has not been identified, it has been suggested that minimal amounts of Tf that are present in the mother can function as a source of iron or that an alternative mechanism for non-Tf-bound iron transfer may exist (Trenor et al. 2000). Holo-transferrin has been shown to bind to transferrin receptors on the maternal membrane of the placenta (King 1976; McArdle et al. 1984). The Tf-TfR complex is then endocytosed in a clathrin-coated vesicle (McArdle et al. 1985b; Srai et al. 2002). The total iron binding capacity was shown to be greater in maternal blood than in umbilical cord blood (Okuyama et al. 1985). Thus, the possibility of transferrin transcytosing the placenta would account for only minimal iron transfer across the placenta (Okuyama et al. 1985; Vanderpuye et al. 1986). The mechanism for transferrin-bound iron processing after endocytosis is similar to that of other cell types. Upon endocytosis, the pH decrease in the endosome causes ferric iron to be released from Tf. Ferric iron is reduced to ferrous iron within the endosome, which can then be
released from the endosome via DMT-1 (Chong et al. 2005; Georgieff et al. 2000; Gruper et al. 2005; Wong et al. 1987). Cytoplasmic ferrous iron can either be stored by ferritin or incorporated into heme and heme-derivative protein within the placenta or it can be released to the fetal system through ferroportin (Blanck et al. 1983; Brown et al. 1979; Donovan et al. 2000; Hodgson and Juchau 1977; Namkung et al. 1983; Okuyama et al. 1985; Starreveld et al. 1995). Similar to hephaestin in the gut, a ferroxidase, zyklopen, exists in the cellular membrane and functions to oxidize the ferrous iron to ferric iron after transfer through ferroportin (Chen et al. 2010). It is mechanistically important to note here that studies evaluating DMT-1 knockdown suggest that the fetus still receives iron. Mice exhibiting a DMT-1 mutation yielded smaller and iron deficient pups that were still viable (Gunshin et al. 2005). Additionally, in a study of Belgrade rats, untreated homozygous females were infertile or their pups failed to thrive (Garrick et al. 1997). Together, these studies indicate that DMT-1 loss does not completely eliminate iron transport across the placenta (Garrick et al. 1997; Gunshin et al. 2005). This suggests the possibility of either an additional exporter from the endosome or an alternative, but unidentified, mechanism by which iron is transported. Recently, hypothesized alternative mechanisms include both heme iron transport and transport through channel proteins such as ZIP8 and ZIP14 (McArdle et al. 2014; Nam et al. 2013; Wang et al. 2012). In a recent review, Cao and O’Brien explore the plausibility of placental heme iron transport, but fail to provide direct evidence to suggest that the process occurs (Cao and O’Brien 2013).
In brief, placental iron transport is completely transferrin-dependent. Transferrin binds to transferrin receptor on the maternal membrane and is endocytosed. The pH decrease in the endosome causes iron to dissociate from transferrin after which it can be transported into the cytoplasm through DMT-1. Once in the cytoplasm, iron can be stored in ferritin, associate with heme, be incorporated into the labile iron pool, or be transported to the fetal circulation through ferroportin. The ferroxidase on the placenta is called zyklopen and functions to convert the ferrous iron released by ferroportin to ferric iron, which is then usable by transferrin.
1.4.2. Regulation of Iron Transport Across the Placenta

Unlike the regulatory process of iron transfer across the enterocytes of the intestine, regulatory mechanisms of placental iron transfer affect iron status of both the mother and the fetus. The fetal liver functions as the primary director of iron regulation and homeostasis for both the fetus and the mother. Studies first showed that as fetal iron absorption increases during gestation and the maternal iron levels decrease, the number of transferrin receptors on the maternal membrane of the placenta also increases (Gambling et al. 2003; McArdle et al. 1985a). Additionally, it was demonstrated that the majority of placental transferrin receptors were located at the maternal membrane (Bradley et al. 2004). In a series of intricately designed experiments, Gambling, et al. demonstrated the ability of the fetal liver to regulate all stages of the maternal iron absorption process (Gambling et al. 2009). They demonstrated that dietary iron availability has direct effects on both maternal and fetal iron status. When fed an iron deficient diet, the maternal hematocrit was maintained through the first half of the pregnancy, but dropped significantly thereafter (Gambling et al. 2009). Furthermore, the iron deficient dams had progressively smaller iron stores in the liver from the beginning of term through the end of gestation (Gambling et al. 2009).

Inspection of gene expression changes revealed as the fetal liver iron increased, the maternal liver transferrin receptor mRNA decreased, but only to a certain level (Gambling et al. 2009). This finding indicates that there may be a threshold to the regulatory system whereby the maternal iron status can only become so deficient. Additionally, analysis of hepcidin mRNA correlations demonstrated that the maternal hepcidin expression strongly correlated with fetal iron levels. When fetal liver iron reached a limit, maternal liver hepcidin became upregulated. As expected, placental transferrin receptor decreased as fetal liver iron increased (Gambling et al. 2009; Martin et al. 2004). These data began to provide evidence for the hierarchical nature of placental iron transport in which the fetal iron level is the priority with the maternal hematocrit being the next
priority and the maternal iron stores being the last priority. This hierarchy concept provides compelling evidence to suggest a potential benefit of iron supplementation throughout pregnancy to aid in maintaining maternal liver iron stores. Despite the fetal ability to alter placental transferrin receptor, studies also suggest that the placenta itself does not change its rate of iron uptake (Bothwell et al. 1958b; Lane 1968; McArdle and Morgan 1982). These studies were performed with fetectomy, though, which evaluated placental iron accumulation for only a short time period. Because these studies do not account for a potentially longer response time after the loss of a fetal regulatory signal, they can only truly provide information regarding short-term adaptations and therefore may not be a true representation of the overall regulatory system (van Dijk 1988).

When taken together, these studies corroborate early studies on the relationship between fetal and maternal iron status. Most early studies tried to determine the effect of pregnancy on maternal iron absorption and the ability of the mother to compensate for deficiency during pregnancy. It was shown that intestinal iron absorption increased during pregnancy, particularly during the third trimester (Apte and Iyengar 1970; Millard et al. 2004; Svanberg 1975). Millard, et al. also demonstrated increased maternal duodenal expression of DMT-1, DcytB, and ferroportin (Millard et al. 2004). These increases were coupled with decreases in hepcidin and transferrin receptor expression in the maternal liver (Millard et al. 2004). The alterations in iron transporter expression supports the ability of the mother to respond to pregnancy-induced iron deficiency to increase iron absorption. The benefit of increased iron absorption appears to be species-dependent, however. In the pregnant rat, it was shown that maternal plasma iron turnover increased six-fold during pregnancy, but that maternal gut iron absorption could account for only 40% of the iron required to meet these demands (Hershko et al. 1976). These data indicate that elevated maternal iron uptake does not meet the requirements of placental iron transfer and
maternal iron stores must also be employed in the process. This observation was not supported by studies in the rabbit, where iron is mobilized from stores to a lesser degree (Bothwell et al. 1958b; van Dijk 1988). The discrepancy is further observed in the human where women may range from no significant decreases in iron stores to severe iron deficiency (Wong and Saha 1990). In studies to evaluate iron supplementation, it was shown that a low-dose resulted in 72% of women depleting their iron stores whereas a higher dose results in 54% of women developing exhausted iron stores (Milman et al. 1991; Thomsen et al. 1993). Despite the varied results seen in mobilization of iron from maternal stores, there is still clear evidence of a maternal iron absorption response to the placental iron transfer depleting maternal hematocrit.

Despite vast evidence regarding the interplay of fetal iron status and placental TfR levels, little evidence exists regarding the regulation of ferroportin export. A study looking at the effects of maternal iron status showed no significant changes in placental ferroportin expression of mild and moderately anemic mothers (Li et al. 2008). This conflicts with cell culture studies in which the BeWo placental cell line was treated with either the iron chelator deferoxamine or with holo-transferrin. When treated with the chelator, ferroportin mRNA levels were increased, but the only significant increase in ferroportin protein was at the highest concentration treatment (Li et al. 2012). Ferroportin was less responsive to iron loading. Ferroportin mRNA was only decreased using a high concentration (12.5 mM) of holo-transferrin and protein was only decreased with both the high concentration and after 72 hours of treatment (Li et al. 2012). The contradictory results in the case of iron deficiency may be reflective of the immortalized nature of a cell line when compared to primary cells or what is seen in vivo. The cell culture chelation studies do support the Gambling, et al. studies previously described in which the placenta responded to iron deficiency and altered its expression profile. It is important to note that while the data were reported as “not shown”, Gambling, et al. do state that no changes in total ferroportin expression
were observed in their study (Gambling et al. 2009). To date, studies have shown that the fetal liver does produce hepcidin, a peptide known to inhibit ferroportin, proportionally to its iron stores (Gambling et al. 2009). Unfortunately, the effect of this increased fetal hepcidin on placental ferroportin remain unclear – while data suggest no changes in placental ferroportin protein expression, studies have yet to address cellular localization of the ferroportin. If the hepcidin induces ferroportin internalization, but does not lead to degradation, this change in cellular localization would still cause less exporter protein at the fetal membrane.

1.5. Iron Transport Across the BBB

The BBB is composed of the microvasculature of the brain. It is characterized by tight junction formation between endothelial cells and complex mechanisms for influx and efflux that tightly regulate the transport of molecules from the blood to the parenchyma of the brain (Abbott 2002; Abbott et al. 2010; Brightman and Reese 1969; Butt et al. 1990). Regulating iron uptake at the BBB is of great importance because too little or too much iron has been associated with various neurological diseases (Table 1-1) (Allen et al. 2001; Connor et al. 1995). Knowledge of the exact mechanism for brain iron uptake was stagnant due to the acceptance of the transferrin transcytosis paradigm established nearly thirty years ago (Figure 1-3, route 1). This model has been favored by those attempting to use transferrin or antibodies to transferrin receptors as a means to deliver therapeutic compounds to the brain (Cabezon et al. 2015; Pardridge et al. 1991; Shin et al. 1995; Walus et al. 1996; Yoshikawa and Pardridge 1992). Transferrin transcytosis was first demonstrated in vivo by evaluating distributions of 125I-Transferrin in the brain and its isolated microvasculature. This study demonstrated that while labeled transferrin remained within the microvasculature, there was also intact transferrin present in the brain supernatant (Fishman et al. 1987). The presence of intact transferrin reaching the brain supports the idea that transferrin, upon
endocytosis by the brain microvasculature, is delivered intact into the brain. Subsequent studies
demonstrated that radiolabeled OX-26, an antibody to transferrin receptor, is capable of binding
brain microvasculature and crossing the BBB into the brain parenchyma in vivo (Friden et al.
1991; Pardridge et al. 1991). Furthermore, it was shown that when OX-26 was conjugated to
proteins, more protein traversed the barrier than when the protein was not conjugated (Shin et al.
1995; Walus et al. 1996; Yoshikawa and Pardridge 1992). Of note, all of these studies
demonstrate retention in the vasculature in addition to detection in the brain.

Subsequent studies, however, have revealed that iron movement into the brain is not solely via
transcytosis. Indeed, the purely transcytosis paradigm for brain iron delivery fails to address a
number of basic biological functions of endothelial cells. First, studies showed that when
unlabeled transferrin or serum were injected in the presence of 125I-transferrin, the amount of
labeled protein seen in the brain fraction decreased (Skarlatos et al. 1995). The decreased uptake
of 125I-Transferrin indicates that this pathway is saturable and dependent upon both serum
transferrin levels and the number of transferrin receptors present on the luminal membrane of the
endothelium. This observation would suggest no regulation of iron uptake into the brain from the
brain side. Moreover, holo-transferrin tightly binds its receptor at physiological pH, which begs
the question—if a transferrin iron complex is transcytosed across the endothelial cell, how is
transferrin or its iron released from the receptor upon reaching the abluminal surface (Klausner et
al. 1983). It has been speculated that a slight environmental pH drop at the abluminal membrane
may allow for detachment but no direct evidence supporting these hypotheses has been
published (Moos and Morgan 2004) and the pH at which Fe begins to dissociate from Tf is ~5.5
(Dauty-Varsat et al. 1983; Tsuno and Sussman 1983) Most importantly, the transferrin
transcytosis model fails to account for the iron needs of the endothelial cells and does not provide
a mode of regulation for iron acquisition by these cells. Given the importance of iron for brain
function and the levels of regulation for iron uptake in the gut and placenta it is difficult to explain or understand how the brain would rely on a simple transcytotic method of iron delivery.

### Table 1-1: Brain disorders associated with brain iron accumulation or brain iron deficiency.

<table>
<thead>
<tr>
<th>Brain Iron Accumulation</th>
<th>Brain Iron Deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurodegeneration with Brain Iron Accumulation (Forni et al. 2008)</td>
<td>Restless Legs Syndrome (Allen and Earley 2001; Connor et al. 2011)</td>
</tr>
<tr>
<td>Hemochromatosis (Bartzokis et al. 2010)</td>
<td>Hypomyelination (Beard and Connor 2003)</td>
</tr>
<tr>
<td>Amyotrophic Lateral Sclerosis (Oba et al. 1993)</td>
<td></td>
</tr>
<tr>
<td>Parkinson’s Disease (Connor et al. 1995)</td>
<td></td>
</tr>
<tr>
<td>Alzheimer’s Disease (Connor et al. 1995)</td>
<td></td>
</tr>
<tr>
<td>Huntington’s Disease (Zecca et al. 2004)</td>
<td></td>
</tr>
</tbody>
</table>

### 1.5.1. Mechanism of Iron Uptake at the BBB

The most commonly accepted model of brain iron uptake involves transferrin (Tf) binding to transferrin receptor (TfR) at the luminal membrane of the endothelial cell (Figure 1-3). The Tf-TfR complex is then endocytosed, at which point opinions differ on the fate of the complex and the iron. The widely established transcytosis mechanism asserts that the Tf-TfR complex, with iron bound to Tf traverses the cell in the endocytosed vesicle. Tf-Fe is then released into the parenchyma at the abluminal membrane. The data for this model, however, are unsatisfactory in
that they fail to address such questions as how the Tf is released from its receptor at the abluminal membrane or more importantly how the endothelial cells comprising the BBB would obtain iron. While there is in vitro evidence for direct transcytosis of Tf bound iron there is also evidence release of non-Tf bound iron indicating that an additional mechanism to transcytosis must be occurring (Burdo et al. 2003). The in vitro data suggesting a disconnect between Tf and iron transfer support in vivo studies in which $^{59}$Fe-$^{125}$I-Transferrin was injected intravenously into rats. These studies demonstrated the presence of non-transferrin bound $^{59}$Fe in the post-capillary fraction, which represented the brain parenchyma after removal of the microvasculature (Moos and Morgan 1998a). The same studies also demonstrated $^{125}$I-transferrin in the post-capillary fraction which could reflect direct transcytosis but it is also possible that this transferrin was taken up through the choroid plexus and distributed into the brain parenchyma. The presence of non-transferrin bound $^{59}$Fe indicates a dissociation of the $^{59}$Fe from the $^{125}$I-transferrin within the endothelial cell before it reached the brain parenchyma which is the model we have put forth (Moos and Morgan 1998a). Pinocytosis remains another potential mechanism that could explain the transport of Tf across the BBB. Pinocytosis has been demonstrated at the BBB, though less frequently than in other microvessels of the body (Baldo et al. 2014; Coomber and Stewart 1986; Smith and Gumbleton 2006; Strazielle and Ghersi-Egea 2013; Zhao et al. 2014). Several studies have, however, shown that inhibiting micropinocytosis does not affect the transport of either Tf or H-ferritin across an in vitro BBB model (Burdo et al. 2003; Fisher et al. 2007). While these studies indicate that pinocytosis does not result in transport of Tf or H-ferritin across the BBB, the inhibitor used only inhibits micropinocytosis and does not, therefore, rule out the possibility of low level pinocytosis contributing to Tf transport at the BBB.
The process by which iron crosses the BBB has recently been modeled (Simpson et al. 2015). Route 1 represents the currently accepted paradigm of the endothelial cell as a passive conduit in which transferrin binds to its receptor on the luminal membrane, traverses the cell and is deposited into the brain. Route 2 represents the more realistic and data-based model in which transferrin is endocytosed after binding to transferrin receptor. The iron is then released by transferrin within the endosome and transported into the cytoplasm through DMT-1. The intracellular iron can be stored in ferritin or it can be released into the brain through ferroportin. This model accounts for the iron needs of the endothelial cells. Route 3 depicts a potential mechanism by which ferritin can transport iron across the BBB. The possibility of this mechanism has been demonstrated, but further study is required to better understand it.
The alternative mechanistic model for brain iron uptake that our laboratory has proposed is that of an endocytic pathway (Figure 1-3, route 2). In this proposed model, upon being endocytosed, the lowered pH of the endosome will cause the release of Fe$^{3+}$ from Tf, which can be reduced to Fe$^{2+}$ by a H-ATPase. The Fe$^{2+}$ is subsequently released into the cytoplasm via DMT-1 where it can be used by the endothelial cell, stored in ferritin, or released into the brain through ferroportin. Upon release, the presence of a ferroxidase such as ceruloplasmin or hephaestin can oxidize the Fe$^{2+}$ and the Fe$^{3+}$ can then be incorporated into apo-Tf circulating in the brain (McCarthy and Kosman 2013; McCarthy and Kosman 2014). One key point of contention with this proposed mechanism is the presence of DMT-1 in the endothelial cells of the BBB. Until recently, there were conflicting studies indicating both the presence and absence of DMT-1 mRNA and protein (Enerson and Drewes 2006; Gunshin et al. 1997; Moos et al. 2006; Siddappa et al. 2003; Siddappa et al. 2002; Skjorringe et al. 2015). The importance of DMT-1 has been demonstrated physiologically in brain iron uptake by the Belgrade rats, which have a mutation that results in non-functioning DMT-1. Belgrade rats have decreased levels of brain iron, which includes decreased levels of microvessel-associated iron (Burdo et al. 1999).

As further support for intracellular release of iron in the endothelial cells forming the BBB, are the reports that endothelial cells of the BBB are capable of storing iron (Simpson et al. 2015). Indeed, the expression levels of ferritin in the BBB are as high as that in the brain per unit protein (Burdo et al. 2004). The BBB acting as an iron reservoir provides evidence against transferrin transcytosis, in which the iron would passively traverse the cell. That endothelial cells are capable of taking up iron and accumulating its own labile iron pool is indicative of an alternative pathway occurring, such as the endocytic mechanism described herein. The endocytic mechanism would result in iron being delivered into the cell’s labile iron pool for storage, usage, or for export,
subsequently explaining how endothelial cells could obtain iron and corroborating the DMT-1 mutation effects on iron transport described previously.

Evidence of alternative mechanisms exists for iron uptake at the level of the BBB, but requires vetting before they are included as actual mechanisms for iron transport across the BBB. While most studies have focused on transferrin-bound iron, both non-transferrin-bound iron and ferritin-bound iron have importance in brain iron uptake. Studies performed in the transferrin-deficient (hpx) mouse, suggest an alternative to the transferrin-dependent mechanism of iron transport at the BBB. In these studies, non-detectable levels of plasma transferrin had only a marginal effect on brain iron uptake except in the choroid plexus. (Beard et al. 2005). Another study examined the effect of the hypotransferrinemia on the brain iron of neonatal mice and found that the hpx mouse actually had significantly more iron in the brain than wild type mice (Takeda et al. 2001). These data suggest that iron uptake by the choroid plexus is Tf dependent and that the redistribution from choroid plexus is Tf dependent. But, the data also suggest that there is an alternative to Tf-mediated iron uptake in the brain because of the generally similar values of iron found in the various regions of the brain of the hpx mice to that of wild type.

In vitro studies demonstrated that H-ferritin not only binds to endothelial cells but also moves across the endothelium of a modeled BBB (Fisher et al. 2007). Fisher, et al. also demonstrated the presence of $^{59}\text{Fe}$ in the brains of rats that received intravenous injection of $^{59}\text{Fe}$-H-ferritin (Fisher et al. 2007). Together, these studies suggest a role for H-ferritin in delivering iron to the brain (Figure 1-3, route 3). Recently, brain microvascular endothelial cells were also shown to take up non-transferrin-bound iron suggesting there is ferrous iron importer at the luminal membrane (McCarthy and Kosman 2014). These cell culture studies are supported by an in vivo study in which mice deficient in transferrin were able to accumulate $^{59}\text{Fe}$ in the brain after injection of
non-transferrin-bound $^{59}$FeCl$_3$ (Malecki et al. 1999). Thus, while transferrin may serve as the primary transporter of iron across the BBB, it seems that the BBB exhibits at least two additional mechanisms by which iron can be taken up into the endothelial cells.

1.5.2. Regulation of Iron Transport at the BBB

As a result of our recently published model for brain transport, regulation of iron transport at the BBB is a bona fide area of investigation. Several studies have focused on the release of iron from the endothelial cell into the brain particularly, the role that ferroxidases play in iron flux from the endothelium of the BBB. It is known that endothelial cells of the BBB, like the enterocytes of the intestine, express hephaestin (McCarthy and Kosman 2013; Patel and David 1997; Qian et al. 2007; Yang et al. 2011). Furthermore, when hephaestin and ceruloplasmin activity was blocked, iron efflux from endothelial cells was also inhibited as the result of ferroportin internalization (McCarthy and Kosman 2013). It was subsequently demonstrated that the addition of soluble ceruloplasmin to cell culture media could reverse the effect of hephaestin and ceruloplasmin knockdown (McCarthy and Kosman 2013). An additional study demonstrated that soluble ceruloplasmin alone could induce iron release from endothelial cells in vitro (McCarthy and Kosman 2013; McCarthy and Kosman 2014). These studies suggest that an exocyttoplasmic ferroxidase may be important, if not crucial to regulating the release of iron from the endothelial cells forming the barrier. Of note, a recent study demonstrated that in the case of hephaestin knockout, brain iron was elevated (Jiang et al. 2015). This study, however, failed to isolate the microvasculature from the whole brain in their analyses and so the increases in iron may be representative of iron accumulation within the BBB rather than increased levels of iron being transported into the brain.
In addition to the ferroxidase studies, the impact of hepcidin on release of iron into the brain has become a prominent area of research. Cell culture studies have demonstrated both directly (Simpson et al. 2015) and indirectly (McCarthy and Kosman 2014) that hepcidin can reduce release of iron from the endothelium. Furthermore, in vivo studies revealed that intracerebroventricular injection of hepcidin had a similar effect, resulting in decreased iron uptake into the brain (Du et al. 2015). The exposure to hepcidin resulted in decreased levels of transferrin receptor, DMT-1, and ferroportin in the brain microvasculature, consistent with the known function of hepcidin to inhibit release and cause iron to accumulate in the endothelial cell (Du et al. 2015). The elevated iron within the cell would explain the decreases in both transferrin receptor and DMT-1.

The effect of various iron management proteins and cerebrospinal fluid (CSF) from monkeys have been evaluated in a model of the BBB. When endothelial cells of a BBB in vitro model are exposed to CSF from iron deficient monkeys, iron release is increased when compared to control CSF (Simpson et al. 2015). Furthermore, when astrocyte-conditioned media was added to the endothelial cells, release was modulated by the iron status of the astrocyte; media from iron deficient astrocytes increased release of iron from the endothelial cell similarly to the CSF from iron deficient monkeys (Simpson et al. 2015). The increased release of iron suggests the presence of a signaling peptide that induces release, but the identity of the peptide remains to be determined. In the same studies, it was shown that apo-transferrin could cause release, providing a candidate for identifying the peptide present in both the iron deficient CSF and the iron deficient astrocyte-conditioned media (Simpson et al. 2015). Lastly, iron-loaded astrocyte-conditioned media had an opposite effect on endothelial cell iron release (Simpson et al. 2015). This identifies the astrocyte as a potential key modulator of iron release from the BBB. It seems highly likely that the astrocyte, which sits at the interface of the endothelium and extends into the
brain parenchyma, could function as an iron sensor for the brain and relay brain iron needs to the
BBB through release of signaling peptides.

Evidence of regulation was demonstrated in an analysis of microvasculature from patients with
Restless Legs Syndrome (RLS) that revealed decreases in transferrin, transferrin receptor, and H-
ferritin (Connor et al. 2011). This study demonstrates the possibility of misregulation of brain
iron transport at the BBB. Because it is known that the brain of a RLS patient is deficient in iron,
one would expect the relevant signals for stimulating iron release to be present. Thus, the
decrease in transferrin receptor and H-ferritin indicate not only an iron deficient endothelium, but
suggests that the BBB is not responding to typical iron cues to upregulate iron transport. Analysis
of iron regulatory proteins (IRP) in the same study further support the concept of regulation at the
intracellular level (Connor et al. 2011). The study confirmed the presence of IRP1 mRNA in the
brain microvasculature and also demonstrated decreased IRP activity within the RLS
microvasculature (Connor et al. 2011). IRPs function to stabilize mRNA of both TfR and DMT-1
when intracellular iron status is low (Eisenstein 2000). The decreased H-ferritin observed in RLS
microvasculature suggests low intracellular iron status, which confounds the observed decrease in
TfR. Thus, the origin of decreased IRP binding activity in RLS brain microvasculature may
provide insights into the cause of brain iron deficiency observed in the diseased RLS brain.

1.6. Importance of the HFE Protein in Barrier Iron Transport

HFE is an iron management protein that has received considerable interest over the last decade in
neurodegenerative diseases. The mutated HFE gene and its protein product are associated with
hereditary hemochromatosis (HH), an autosomal-recessive iron overload condition (Beutler et al.
2001; Connor and Lee 2006). After the HFE gene was identified, two mutations, C282Y and
H63D, were linked to HH (Feder et al. 1996). The C282Y gene variant has a lower general population prevalence of 1.9% compared to 8.1% for the H63D variant, but the C282Y variant is more commonly associated with HH (Merryweather-Clarke et al. 1997). The wildtype HFE protein functions through its association with both β2 microglobulin (β2M) and Tfr (Feder et al. 1998; Feder et al. 1997; Waheed et al. 1997). The interaction with β2M directs HFE to the cell membrane, allowing for HFE to interact with Tfr (Feder et al. 1997; Waheed et al. 1997). The interaction with Tfr regulates iron uptake by allowing for Tfr to associate with only one Fe-Tf molecule (Feder et al. 1998; Lebron et al. 1999). When mutated, the association of HFE to Tfr is lost, resulting in increased Fe-Tf uptake into cells (Feder et al. 1998; Lebron et al. 1999). Bastin, et al. showed HFE staining in the placenta, the crypt cells of the gut, and the brain endothelium, which was supported by additional studies demonstrating the presence of HFE in cortical blood vessels (Bastin et al. 1998; Connor et al. 2001b). Because of mounting evidence that the presence of HFE mutations may increase risk or alter the course of many neurodegenerative diseases it became important to understand how the HFE protein is functioning to modulate iron transport at the brain but also other physiological barriers (Altamura and Muckenthaler 2009; Loeffler et al. 1995; Zecca et al. 2004).

Several studies have been performed to understand the role that HFE gene variants play in modulating iron absorption at the gut. The studies, however, have yielded mixed results from which the importance of HFE is difficult to interpret. It was shown that deletion of the HFE gene resulted in increased levels of $^{59}$Fe absorption as well as an increase in the expression of DMT-1 mRNA in the duodenum (Bahram et al. 1999; Fleming et al. 1999a). These data are suggestive of HFE playing a crucial role in regulating iron absorption and it was proposed that HFE participates in systemic iron sensing at the crypt cell. Another study, however, failed to detect changes in DMT-1 protein in a mouse model of iron overload that resembles HH (Canonne-Hergaux et al.
Later human studies revealed that patients with HH (C282Y) did not exhibit decreases in duodenal DMT-1 or FPN gene expression when compared to normal controls (Stuart et al. 2003). There is clearly a role for HFE in maintaining systemic iron levels but the mechanism remains unclear and much of the current research has focused on an apparent misregulation of hepcidin resulting from the mutation in HFE (Goswami and Andrews 2006; Nemeth 2008; Piperno et al. 2007; Schmidt et al. 2008).

The role for HFE in the placenta is even less well-elucidated. Studies to determine HFE localization in the placenta yielded mixed results. Earlier work revealed highly prevalent HFE staining on the apical membrane, which is also very TfR1 dense (Parkkila et al. 1997). In a later study, however, Bastin et al. demonstrated that HFE was actually more prevalent on the basolateral membrane (Bastin et al. 2006). Furthermore, this study demonstrated colocalization of HFE with FPN rather than TfR1, which suggests an alternative role for HFE in the placenta (Bastin et al. 2006). More recently, it was shown that knockout of HFE in the mother resulted in elevated iron status in the fetus as well as increased expression of TfR1, DMT-1, and FPN in the placenta, which supports a crucial role for HFE in regulating iron transport across the placenta (Balesaria et al. 2012).

For many years, HFE mutations were not thought to impact the brain because of the BBB. This concept was based on erroneous interpretations of two autopsy-based studies in which it was reported there was more iron in the brain in areas not protected by the BBB (Cammermyer 1947; Sheldon 1935). The studies both reported, which failed to get attention in the literature, that there was more iron also in areas behind the BBB (Cammermyer 1947; Sheldon 1935). Subsequent MRI studies (Berg et al. 2000; Nielsen et al. 1995; Rutgers et al. 2007) and a mouse model of the most abundant HFE gene variant (Nandar et al. 2013) report elevated iron in the brain. Studies
have shown that HFE protein does exist in the brain endothelium, but the function has not been determined (Bastin et al. 1998; Connor et al. 2001a); although there is no reason to suspect it functions any differently in the BBB than in other cells. Several studies have demonstrated elevated brain iron, modified white matter myelination, and increased risk for developing neurodegenerative disease in the presence of HFE mutations (Berlin et al. 2004; Meadowcroft et al. 2015; Nandar et al. 2013; Pulliam et al. 2003). Thus, understanding if and how HFE alters brain iron uptake may help to address questions regarding elevated brain iron and the subsequent risks for disease in the presence of HFE gene mutations.

1.7. Summary of the Similarities of Gut and Placenta Iron Uptake Mechanisms to BBB Iron Uptake Mechanisms

The purpose of this review was to compare and contrast what is understood about iron transport at other barriers in the body to that in the brain. Current advances in brain iron uptake studies have demonstrated similarities and differences between the roles of iron management proteins as observed in both the intestine and the placenta (Figure 1-4). When comparing overall mechanistic schemes, recent BBB studies seem to indicate a higher level of similarity to placental iron transport. Both mechanisms appear to be primarily transferrin-dependent, although the BBB remains distinct in that it evokes both a transcytotic and an endocytic mechanism. Additionally, the endocytic freeing of iron from transferrin and release of ferrous iron into the cytoplasm by DMT-1 appear to be the same at both barriers. However, DMT-1 mutation studies demonstrate a key connection between all three barriers – that additional modes of iron transport must exist in these systems.
Figure 1-4: Similarities and differences in iron transport at physiological barriers When comparing iron transport at the gut, the placenta, and the BBB, there are three key similarities seen at all barriers. First, each barrier appears to demonstrate presently unidentified alternative transport mechanisms. Secondly, DMT-1 is involved in iron trafficking at each barrier. Finally, all three barriers also utilize ferroportin on their basolateral membrane for iron export. Evaluation of each overall mechanism suggests that the BBB is most similar to the placenta. Iron transport at both barriers is primarily transferrin-dependent and involves a mechanism by which iron is released from transferrin in the endosome for export into the intercellular iron pool. Unlike either of the other barriers, the gut requires uptake of non-transferrin bound iron. The BBB appears to be unique in its ability to transcytose transferrin-bound iron. Additionally, the potential for heme transport has been hypothesized in both the placenta and the gut, but there is no evidence to suggest that this occurs at the BBB. In terms of regulation, the BBB demonstrates transferrin-transferrin receptor feedback signaling that is similar to what is observed in the crypt cells of the gut. Meanwhile, the placental regulatory system is unique in that iron transport is under the control of both the mother and the fetus.
Furthermore, the presence of regulatory mechanisms at both the gut and placenta substantiate the hypothesis that regulatory mechanisms exist at the BBB. The BBB shares mechanistic similarities with the placenta for uptake and transport, but regulation of brain iron uptake appears to more closely resemble that of the gut. Transferrin receptors have been demonstrated on both the luminal and abluminal membranes of the BBB (Huwyler and Pardridge 1998; Simpson et al. 2015). The presence of transferrin receptors on the abluminal surface of the BBB could indicate a similar feedback mechanism for regulation as seen in the crypt cells of the intestine. We propose that the Tf receptors on the abluminal membranes sense iron status in the brain, much like transferrin receptors sense systemic iron status in the gut through the amount of saturated Tf. If this is the case, during brain iron sufficiency, holo-transferrin could be feeding back into the endothelial cell. The feedback system might result in an increase in intracellular iron, which would decrease luminal transferrin receptor expression and, subsequently, decrease iron uptake.

Moving forward, elucidating the iron transport system at the BBB holds potential significance for the field of drug delivery across the BBB. A long-standing theory has been that the transferrin transcytosis pathway can be exploited to improve drug delivery into the brain (Pardridge et al. 1991; Shin et al. 1995; Walus et al. 1996; Yoshikawa and Pardridge 1992). Unfortunately, this targeting system has been mostly unsuccessful. The approach to using Tf or antibodies to Tf receptors to circumvent the BBB for drug delivery failed to consider that transferrin receptor-targeted compounds end up in the late endosome or lysosome rather than being transported directly into the brain (Cabezon et al. 2015). Moving forward, understanding the complete mechanistic and regulatory processes by which iron, and transferrin, cross the BBB and regulation of the pathways for transcytosis versus endocytosis will be of utmost importance in helping to explain a critical nutrient and key factor of multiple brain diseases as well as potentially helping to more effectively deliver drugs to the brain.
1.8. References

Bedard YC, Pinkerton PH, Simon GT (1976) Uptake of circulating iron by the duodenum of normal mice and mice with altered iron stores, including sex-linked anemia: high resolution radioautographic study Laboratory investigation; a journal of technical methods and pathology 34:611-615


Bothwell TH, Pribilla WF, Mebust W, Finch CA (1958b) Iron metabolism in the pregnant rabbit; iron transport across the placenta The American journal of physiology 193:615-622


Brown PJ, Johnson PM, Ogbimi AO, Tappin JA (1979) Characterization and localization of human placental ferritin The Biochemical journal 182:763-769


Feder JN et al. (1996) A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis Nature genetics 13:399-408 doi:10.1038/ng0896-399

Feder JN et al. (1998) The hemochromatosis gene product complexes with the transferrin receptor and lowers its affinity for ligand binding Proceedings of the National Academy of Sciences of the United States of America 95:1472-1477


Fleming RE et al. (1999b) Mechanism of increased iron absorption in murine model of hereditary hemochromatosis: increased duodenal expression of the iron transporter DMT1 Proceedings of the National Academy of Sciences of the United States of America 96:3143-3148


Garrick M et al. (1997) Iron supplementation moderates but does not cure the Belgrade anemia Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine 10:65-76


Green R et al. (1968) Body iron excretion in man: a collaborative study The American journal of medicine 45:336-353

Gruper Y, Bar J, Bacharach E, Ehrlich R (2005) Transferrin receptor co-localizes and interacts with the hemochromatosis factor (HFE) and the divalent metal transporter-1 (DMT1) in trophoblast cells Journal of cellular physiology 204:901-912 doi:10.1002/jcp.20349


Huggenvik JI, Craven CM, Idzerda RL, Bernstein S, Kaplan J, McKnight GS (1989) A splicing defect in the mouse transferrin gene leads to congenital atransferrinemia Blood 74:482-486


Lane RS (1968) Regulating factors in the transfer of iron across the rat placenta British journal of haematology 15:365-369


Moos T, Morgan EH (1998b) Kinetics and distribution of [59Fe-125I]transferrin injected into the ventricular system of the rat Brain research 790:115-128


Parkkila S et al. (1997) Association of the transferrin receptor in human placenta with HFE, the protein defective in hereditary hemochromatosis. Proceedings of the National Academy of Sciences of the United States of America 94:13198-13202


Takeda A, Takatsuka K, Connor JR, Oku N (2001) Abnormal iron accumulation in the brain of neonatal hypotransferrinemic mice Brain research 912:154-161


Waheed A et al. (1997) Hereditary hemochromatosis: effects of C282Y and H63D mutations on association with beta2-microglobulin, intracellular processing, and cell surface expression of the HFE protein in COS-7 cells Proceedings of the National Academy of Sciences of the United States of America 94:12384-12389


Wong CT, McArldle HJ, Morgan EH (1987) Effect of iron chelators on placental uptake and transfer of iron in rat The American journal of physiology 252:C477-482

Wong CT, Saha N (1990) Inter-relationships of storage iron in the mother, the placenta and the newborn Acta obstetricia et gynecologica Scandinavica 69:613-616


Yoshikawa T, Pardridge WM (1992) Biotin delivery to brain with a covalent conjugate of avidin and a monoclonal antibody to the transferrin receptor The Journal of pharmacology and experimental therapeutics 263:897-903
doi:10.1038/nrn1537
Chapter 2

The Role of the Blood-Brain Barrier in Maintaining Brain Iron Status

2.1. Abstract

The mechanism for brain iron uptake has recently been expanded to include a mechanism that allows for free iron being exported from the endothelium of the blood-brain barrier (BBB). Furthermore, the new paradigm establishes the BBB as the key site for regulation of brain iron uptake. Here, we demonstrate that endothelial cells of the BBB are capable of retaining and releasing iron in response to signaling peptides in an in vitro BBB model. Our studies indicate that iron-poor transferrin (apo-Tf) and an iron chelator, deferoxamine (DFO), stimulate release of iron from iron-loaded endothelial cells. Furthermore, inhibition of the iron exporter, ferroportin, by hepcidin does not cause a significant decrease in release of iron from the cells alone or in the presence of apo-Tf. Although transport of iron across and release of iron from cells forming the BBB are important aspects of brain iron delivery, uptake of iron into the endothelial cells is critical because the endothelial cells require iron for their metabolic needs. Divalent metal transporter 1 (DMT-1) has been hypothesized to function in the endosome to release free iron into the endothelial cell. Here, we show that while there were no significant changes in the total iron or transferrin transported across the BBB in the presence of a DMT-1 inhibitor, the ratio of protein-bound iron to free iron was reduced. An altered ratio of protein-bound to free iron suggests that iron movement across the endothelium may have been shifted to the recycling stage of the pathway. These data substantiate the recently hypothesized endocytic mechanism by demonstrating a role for DMT-1 in iron transport across endothelial cells.
2.2. Introduction

Iron is an essential micronutrient that functions as a cofactor in key brain processes such as myelination and neurotransmitter synthesis (Beard et al. 1996). The mechanism by which the brain accumulates iron has received relatively little attention which is surprising given the contribution of altered brain iron status observed in various neurodegenerative diseases (Allen et al. 2001; Beard and Connor 2003; Connor et al. 2011; Connor et al. 1995; Forni et al. 2008; Oba et al. 1993; Zecca et al. 2004) and the attempts to use the iron delivery system to the brain to deliver therapeutic agents (Pardridge 1988; Pardridge et al. 1991; Shin et al. 1995; Skarlatos et al. 1995; Walus et al. 1996; Yoshikawa and Pardridge 1992). Until recently, studies on the uptake mechanism of iron into the brain considered the blood-brain barrier (BBB) as a passive conduit.

A key aspect to understanding the BBB as the regulatory site for brain iron uptake has to include a model wherein the iron needs of the endothelial cells are met. Until recently, the fact that mitochondrial rich (Kemper et al. 2013; Oldendorf et al. 1977; Razmara et al. 2008), ferritin-containing (Burdo et al. 2004) endothelial cells had a mechanism for acquiring iron and not just transporting it had been ignored. Our laboratory and others have now identified a potential endocytic mechanism (McCarthy and Kosman 2013; McCarthy and Kosman 2014; Simpson et al. 2015) that is similar to all cell types where transferrin-bound iron (TBI) is endocytosed and then iron is released into the cytoplasm through divalent metal transport 1 (DMT-1) (Fleming et al. 1998; Trinder et al. 2000). This iron can then be used for the metabolic needs of the endothelial cells or stored in the cells in ferritin (Burdo et al. 2004). Importantly, the presence of the iron exporter, ferroportin, has been confirmed at the membrane of brain microvascular endothelial cells, but its function has not been confirmed (McCarthy and Kosman 2013; Wu et al. 2004; Yang et al. 2011). A role for ferroportin has been implied in release of iron to the brain from endothelial
cells, but not demonstrated directly, by utilizing an inhibitor of hepcidin, the peptide known to block iron export through ferroportin. (McCarthy and Kosman 2013; McCarthy and Kosman 2014). We have also shown that the iron stored in the endothelial cells can be exported but we have not identified the mechanism (Simpson et al. 2015).

In this study, we identify factors that alter release and transport of iron across a model of the BBB. Specifically, we investigate the regulation of ferroportin by hepcidin on release and demonstrate a role for DMT-1 in movement of iron across the BBB. Our previous studies have shown that there are at least two mechanisms by which iron gets into the brain: 1) direct transcytosis bound to Tf and 2) release of iron that had been stored in the endothelial cells (Simpson et al. 2015). The latter appears to be responsive to signals from the brain (i.e. cerebrospinal fluid (CSF) and extracellular fluid) while the direct transcytotic pathway appears to be constitutive. Here, we examined the mechanisms associated with iron release from the endothelial cells and examined additional factors to further interrogate the transcytotic pathway. Together, our previous data and the data presented here substantiate the presence of signaling and regulation of brain iron uptake at the level of the BBB. Importantly, this study implicates DMT-1 as a key mediator of brain iron transport.

2.3. Materials and Methods

2.3.1. Radiolabeling of Transferrin

$^{59}$Fe (40 uCi, Perkin Elmer) was complexed with 40 uL nitrilotriacetic acid (NTA), 0.5 uL 1 mg/mL ferric chloride, and 2 uL 0.5 M sodium bicarbonate to form an $^{59}$Fe-NTA complex. After
incubating apo-Tf (1.2 mg, Sigma) with the $^{59}\text{Fe}$-NTA complex, free iron was separated from the
$^{59}\text{Fe}$-Tf using a Sephadex-G25 QuickSpin column as per the manufacturer’s instructions (Roche).

2.3.2. Cell Culture

Bovine brain microvascular endothelial cells (BBMVEC) were cultured in complete growth
medium (Cell Applications, Inc.). An in vitro model of the BBB was used to perform functional
assays. BBMVECs were grown to confluence on Costar transwell 0.4 µm porous filters (Corning)
and then gently washed three times with 1X DPBS (Corning). A treatment of serum free media
containing 138 nM hydrocortisone for 72 hours was then used because hydrocortisone
supplementation induces the formation of tight junctions (Antonetti et al. 2002; Kurzen et al.
2002).

2.3.3. Release Assay

BBMVECs in the transwell setup were loaded overnight with 10 µCi/well of $^{59}\text{Fe}$-NTA complex.
Wells were then washed three times with 1X DPBS (Corning) to remove iron remaining in the
culture medium. Serum-free medium containing 70 kDa RITC-Dextran (Sigma Aldrich) was
added to the apical chamber. The 70 kDa RITC-Dextran functions as a measure of barrier
integrity in this model as this protein has been shown to not cross the BBB freely. Serum-free
medium containing 10 uM apo-Tf (Sigma Aldrich), 500 nM hepcidin (Peptides, Inc.), or apo-Tf
and hepcidin was then added to the basal chamber. Where hepcidin was used, BBMVECs were
treated with 500 nM hepcidin overnight simultaneously with the $^{59}\text{Fe}$ loading step. Data were
collected by taking 50 uL aliquots from the apical chamber at 0 and 6 hours and the basal
chamber at 0, 2, 4, and 6 hours. Fluorescence of the samples was measured on a SpectraMax Gemini EM plate reader (Molecular Devices) and $^{59}\text{Fe}$ was then measured on a Beckman Gamma 4000 (Beckman Coulter).

### 2.3.4. Transport Assay

Tf flux across the in vitro BBB was measured by applying $^{59}\text{Fe}$-AlexaFluor 488-Tf to the apical chamber of our transwell model. Fresh media containing the $^{59}\text{Fe}$-AlexaFluor 488-Tf was added to the apical chamber at the beginning of the experiment with 70 kDa RITC-Dextran to monitor barrier permeability. For this transport study, apo-Tf treatment used in the release study was repeated to determine its effect on transport across the barrier rather than release. Additionally, the role of DMT-1 in transport of iron across the BBB was studied. For these assays, a DMT-1 inhibitor (XEN602, Xenon Pharmaceuticals) was added to the apical chamber for 1 hour before $^{59}\text{Fe}$-AlexaFluor 488-Tf was added at the 0-hour time point. We tested XEN602 in the presence and absence of apo-Tf in an attempt to elucidate the pathway through which apo-Tf signals. For all conditions, media aliquots were taken from the apical chamber at the 0-hour time point and the end point. Basal chamber aliquots were taken at 2-hour intervals over a 6-hour time course. Fluorescence was measured in all samples on a SpectraMax Gemini EM plate reader (Molecular Devices) and $^{59}\text{Fe}$ was measured on a Beckman Gamma 4000 (Beckman Coulter). A Sephadex G-25 Quick Spin column was used as per the manufacturer’s instructions (Roche) to separate free iron from protein-bound iron.
2.3.5. Protein Expression Assay

Ferroportin protein expression was measured by western blotting. Cells were lysed in 70 µL of RIPA lysis buffer containing 1X protease inhibitor cocktail (Sigma Aldrich), pooling three wells per sample. 10 µg of protein was separated on a 4-20% Criterion TGX gel by SDS-PAGE gel electrophoresis. The protein bands were then transferred to Amersham Hybond PVDF membrane (GE Healthcare Life Sciences). The membranes were blocked in 5% milk for 1 hour at room temperature. The membrane was then exposed to ferroportin primary antibody (1:200, Alpha Diagnostic International) overnight at 4°C. The membranes were washed three times using 1X TBS-Tween (0.05 M Tris Base, 0.15 M NaCl, 0.05% Tween-20, pH 8.0) for 5 minutes per wash. The membranes were then incubated for 1 hour at room temperature with anti-mouse secondary antibody (1:5000, GE Healthcare Life Sciences). After three 10-minute washes, the membranes were visualized by adding Western Lightning Plus-ECL (Perkin Elmer) and imaged on a Fuji LAS-3000 Imaging System. Densitometry was performed using Multigauge software (Fuji Film). β-actin (1:3000, Sigma Aldrich) was used as a loading control. To control for cross-membrane variability, a common liver sample was used on each blot for normalization.

2.3.6. Gene Expression Assay

Total RNA was isolated from BBMVECs cultured under the conditions studied in both release and transport assays using the Qiagen RNEasy isolation kit (Qiagen). Total RNA was detected by the Nanodrop ND-1000 spectrophotometer. The Applied Biosystems High Capacity cDNA kit (Applied Biosystems by Thermo Fisher Scientific) was used to synthesize cDNA. Quantitative real-time polymerase chain reaction (qPCR) was performed using the Taqman Gene Expression
Assay (Applied Biosystems by Thermo Fisher Scientific). Probes for the genes assessed is described in Table 2-2. Relative mRNA levels were corrected relative to an internal cDNA standard and were then normalized to a reference gene, Ribosomal protein S9 (RPS9).

Table 2-1: TaqMan qPCR Probes.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>TaqMan Probe Assay ID</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFRC</td>
<td>Bt04296079_m1</td>
<td>Iron uptake</td>
</tr>
<tr>
<td>FTL</td>
<td>Bt03216295_g1</td>
<td>Iron storage</td>
</tr>
<tr>
<td>FTH</td>
<td>Bt03244348_g1</td>
<td>Iron storage</td>
</tr>
<tr>
<td>SLC40A1</td>
<td>Bt02810822_m1</td>
<td>Iron export</td>
</tr>
<tr>
<td>RPS9</td>
<td>Bt03272017_m1</td>
<td>Ribosomal protein, protein synthesis</td>
</tr>
</tbody>
</table>

2.3.7. Statistical Analyses

Prism (GraphPad Software) software was used for all statistical analyses and data graphing. Data from three independent replications were pooled and are expressed as mean ± standard error of the mean. Statistical differences between experimental groups were determined using an unpaired Student’s t test, one-way ANOVA and Bonferroni’s multiple comparisons test, or two-way ANOVA and Bonferroni’s multiple comparisons test. A level of significance of p < 0.05 was used for all differences evaluated.
2.4. Results

2.4.1. Regulation of Iron Release in an In Vitro BBB Model

In the first series of studies, we determined the ability of hepcidin to block iron release from the endothelial cells. The BBMVEC were loaded with $^{59}$Fe and then exposed to hepcidin for 4 hours. There was no effect on the baseline release of $^{59}$Fe from the cells (Figure 2-1A). Exposure to apo-Tf on the abluminal (CSF side) of the BBMVEC resulted in a 140% increase in iron release within 2 hours of exposure that persisted throughout the time periods examined (Figure 2-1C). The apo-Tf release was not blocked by hepcidin exposure (Figure 2-1B). The action of hepcidin is through the iron export protein, ferroportin. Therefore, we determined that ferroportin is expressed in the BBMVEC (Figure 2-2) and its expression is decreased 38% with iron loading. The addition of hepcidin or apo-Tf had no effect on the ferroportin expression when they were added alone to the iron loaded endothelial cells but when added in combination the levels of ferroportin were the same as control indicating that the combination of hepcidin and apo-Tf could block the iron induced decrease in ferroportin.
Figure 2-1: Iron release from BBMVECs in a modeled BBB after exposure to 10 uM apo-Tf, 100 uM DFO, and 500 nM hepcidin. A) 500 nM hepcidin treatment had no significant effect on $^{59}$Fe release from BBMVEC. B) 10 uM apo-Tf significantly increased release into the basal chamber from BBMVEC. The apo-Tf induced iron release was not altered by 500 nM hepcidin treatment. C) Apo-Tf, a physiologically-relevant iron chelator, and DFO, a pharmacologic iron chelator, cause significant release of iron into the basal chamber over 4 hours. Concentrations were selected based on documented standard in vitro conditions. Of note, 100 uM DFO can bind 5 times more iron than 10 uM apo-Tf. Data are represented as mean ± SEM, n = 3 per condition. *p < 0.05, ***p < 0.001, ****p<0.0001.
**Figure 2-2:** Effect of treatment with Fe-NTA, apo-Tf, and hepcidin on ferroportin protein expression in BBMVECs. A) Representative western blot of ferroportin and β-actin. B) There was a consistent decrease of 38% in ferroportin when BBMVECs were treated with Fe-NTA and this decrease was not affected by exposure to 500nM hepcidin. The decrease in ferroportin in response to apo-Tf was eliminated when the cells were treated with a combination of Fe-NTA, apo-Tf and hepcidin. Ferroportin was expressed as a ratio of the protein to β-actin in the endothelial cells. The ratio was normalized to a common liver sample to account for intra-experimental variance. Data are represented as mean ± SEM, n = 3 per condition. *p < 0.05.
The mRNA expression of select iron management proteins was measured because their translation is regulated by iron levels (Figure 2-3) (Eisenstein 2000). Hepcidin exposure resulted in a 58% increase in ferroportin mRNA in the untreated, unloaded control condition (Figure 2-3A). Additionally, iron loading the BBMVEC yielded an 86% increase in ferroportin mRNA compared to the unloaded control (Figure 2-3A). When the BBMVEC were treated alone with hepcidin or were only pre-loaded with iron, there was no significant effect on TfR mRNA observed. However, when combined, the iron loading of the endothelial cell combined with the hepcidin exposure resulted in a 36% decrease in TfR mRNA (Figure 2-3B). The mRNA expression of the L and H subunits of the iron storage protein, ferritin, was not altered (Figure 2-3 C&D).
Figure 2-3: mRNA expression in BBMVEC after treatment with Fe-NTA, apo-Tf, and hepcidin. A) There was an overall trending increase in ferroportin mRNA expression in BBMVECs. Hepcidin alone caused a 58% increase and iron loading caused an 86% increase in ferroportin mRNA. B) There was a trending decrease in transferrin receptor expression. Specifically, hepcidin in the presence of iron resulted in a 36% decrease in transferrin receptor mRNA levels. C) L-ferritin mRNA was not affected by release treatments. D) H-ferritin mRNA levels remained consistent in the presence of the release condition treatments. Data are represented as mean ± SEM, n = 3 per condition.
2.4.2. Mechanisms and Regulation of Iron Transport in an *In Vitro* BBB Model

A critical question for brain iron transport studies is the role of DMT-1. To investigate the potential role of DMT-1 in $^{59}\text{Fe}$ transport, we exposed the BBMVECs to a DMT-1 inhibitor, XEN602. The presence of XEN602 had no effect on $^{59}\text{Fe}$ transport (Figure 2-4A) or the amount of iron remaining in the cell lysates (Figure 2-4B). The rate of Tf transport was measured using fluorescently labeled Tf and was not significantly altered XEN602 (Figures 2-4C). There were changes, however, in the ratio of bound to non-bound iron that was transported in response to exposure to XEN602 (Figure 2-4D). Specifically, both protein-bound $^{59}\text{Fe}$ (Figure 2-4E) and unbound $^{59}\text{Fe}$ (Figure 2-4F) were reduced after XEN602 treatment.
Because we demonstrated that apo-Tf could increase release of iron from BBMVEC, we sought to determine if it would also impact iron transport. We demonstrated that apo-Tf in the basal chamber results in a 33% (but statistically insignificant) decrease in $^{59}$Fe transport (Figure 2-5A). Exposure to apo-Tf had no impact on $^{59}$Fe retention within the BBMVEC (Figure 2-5B) or Tf flux into the basal chamber (Figure 2-5C). In this study, we also exposed the BBMVECs to
XEN602 and apo-Tf simultaneously to determine the if route of transport potentially affected by apo-Tf involved DMT-1. We found that XEN602 had no significant effects on $^{59}$Fe transport or Tf flux in the presence of apo-Tf (Figures 2-5A & 2-5C). Neither apo-Tf alone nor apo-Tf in conjunction with XEN602 altered protein-bound $^{59}$Fe flux (Figure 2-5D), but we did observe a significant reduction in free $^{59}$Fe transported in these conditions (Figure 2-5E). Consequently, the ratio of bound-to-unbound $^{59}$Fe was increased by 327% in response to apo-Tf alone and 292% in response to apo-Tf and XEN602 (Figure 2-5F).
mRNA levels for iron management proteins were measured to determine if the altered ratio of protein-bound to unbound iron could be due to secreted proteins from the BBMVEC. If mRNA for Tf or ferritin is elevated, the endothelial cells could be secreting proteins in response altered iron status from the experimental conditions. Treatment with apo-Tf in the transport model
resulted in a 20% decrease in ferroportin mRNA expression (Figure 2-6A). TfR mRNA levels were decreased 29% in the presence of apo-Tf and 15% when exposed to XEN602 with or without apo-Tf (Figure 2-6B). No significant differences were observed in H-ferritin mRNA, but there was an average 23% decrease in L-ferritin mRNA levels across all conditions (Figure 2-6C&D).

**Figure 2-6**

**Figure 2-6:** mRNA expression in BBMVEC after treatment with apo-Tf and XEN602 alone or apo-Tf and XEN602 combined. A) Ferroportin was decreased by 20% with apo-Tf treatment. B) There was a 29% decrease in transferrin receptor mRNA expression after BBMVEC exposure to apo-Tf and a 15% decrease with XEN602. C) L-Ferritin mRNA was, on average, decreased by 23%. D) H-Ferritin mRNA was not affected by transport study treatments. Data are represented as mean ± SEM, n = 3 per condition.
2.5. Discussion

The results of this study reveal that iron is stored in endothelial cells and the release of iron can be induced by factors from the brain. Additionally, the results implicate DMT-1 as a key transporter in the endocytic mechanism for brain iron uptake. The studies with the DMT-1 inhibitor suggest that the data originally interpreted as a transcytotic mechanism may better fit a model of recycling of endosomal diferric Tf to both membranes of the endothelium.

We previously reported that the BBB was capable of storing iron based on observations of ferritin (for iron storage) and iron regulatory proteins (for regulation of ferritin, transferrin receptor, ferroportin) in blood vessels isolated from rodent and human brains (Simpson et al. 2015). More recently, we have demonstrated retention of iron in the endothelial cells of the brain (manuscript under review). A key protein for the endocytic mechanism of iron uptake and release of iron into the endothelial cell cytoplasm is DMT-1. Here, we incorporated a DMT-1 inhibitor into our model to evaluate the role of DMT-1 in iron transport at the BBB. In both the presence and absence of apo-Tf, the inhibitor had no effect on total transferrin or total iron transported across the in vitro endothelium. The presence of DMT-1 inhibitor, however, altered the ratio of protein-bound to free iron that was transported across the cultured endothelium (Figure 2-7).
While we have previously demonstrated that blocking endocytosis with NH$_4$Cl also alters this ratio (Burdo et al. 2003), the changes observed in the present study are not consistent with our previous studies. In our previous study, the endocytosis blocker resulted in decreased free iron, but the amount of bound iron was not altered. In the current study, however, we demonstrate...
decreases in both bound and unbound iron when treated with the DMT-1 inhibitor, XEN602. The difference in our results can be explained by pH of the endosomes and provides significant insights into the mechanism of iron transport across the model of the BBB. NH₄Cl inhibits the endocytic pathway by blocking H-ATPases, which blocks the acidification of the endosome (Carpenter and Cohen 1976). XEN602 is specific to the iron transport process, only functioning to block the exit of ferrous iron via DMT-1 (Cadieux et al. 2012). In studying of the transferrin cycle in reticulocytes from the Belgrade rat, which expresses mutated DMT-1, the mutated reticulocyte exocyted 80% of iron bound to transferrin compared to 45% in the normal reticulocyte (Garrick et al. 1993). In the reticulocyte, the TBI is recycled back to the cell membrane from which it originated. Therefore, we believe that by blocking DMT-1 in the endosome of our BBMVEC, recycling is forced towards the luminal membrane from which endocytosis occurs and limits distribution of the vesicle to the abluminal membrane. The recycling of all TBI to the luminal membrane would explain the decreased protein-bound iron detected in the basal chamber shown here. This observation expands the current model by providing a new interpretation of previous data. Unlike other cells, the endothelium of the BBB is polarized and therefore has two sides of the cell to which vesicles could be recycled. Rather than the transcytosis previously invoked to account for the transport of Tf bound iron, we could, in fact, be detecting recycling to both the apical and basal chamber rather than recycling solely to the apical chamber.

In addition to recycling of endosomes to both luminal and abluminal membranes, the cells of the BBB can store iron as we have previously shown (Simpson et al. 2015). This iron can be released in response to apo-Tf and DFO and conditioned media from iron deprived astrocytes (Simpson et al. 2015). We hypothesized the mechanism for iron release from the endothelial cells involved export via ferroportin. In this study, exposure to hepcidin did not affect the release of iron
induced by apo-Tf, yet ferroportin expression was decreased following iron loading. The decrease in ferroportin expression following iron loading did not occur when hepcidin was added with apo-Tf. Thus similar amounts of iron were released in the presence of apo-Tf despite the presence of hepcidin and significantly different levels of ferroportin expression. This finding is different from our previous report using bovine retinal endothelial cells where hepcidin exposure resulted in a moderate decrease in $^{59}$Fe release and a slight decrease in ferroportin expression (Simpson et al. 2015) suggesting there are differences in ferroportin expression and the response to hepcidin between the retinal and brain microvasculature. Because ferroportin must be located in the cellular membrane to be functional as an exporter, the values measured in total lysate may not properly inform on the iron management profile of the BBMVEC. Indeed, the ferroportin mRNA increased 58-86% in the iron loaded cells where the protein was decreasing. The increase in message is consistent with elevated intracellular iron loading because of the presence of an iron responsive element in the 5’ untranslated region of ferroportin (Abboud and Haile 2000; Liu et al. 2002; Lymboussaki et al. 2003; McKie et al. 2000). The level of ferroportin in the apo-Tf/hepcidin treatment condition relative to the iron loaded only condition coupled with the normal iron release data suggests ferroportin on the membrane could be similar in the two conditions due to simultaneous protein degradation and synthesis. We cannot rule out that there are alternative mechanisms for iron release.

This study, in conjunction with our previous findings, provides new insights into brain iron transport and its regulation. Our model shows how changes in levels of apo-Tf can increase iron release and the changes in amount of apo-Tf could occur in a microenvironment as a function of iron uptake by neurons and recycling of apo-Tf from holo-Tf. Furthermore, the studies using XEN602 solidify the role of DMT-1 in brain iron uptake. This finding supports the presence of the hypothesized endocytic mechanism for iron movement across the BBB. In addition to
confirming the endocytic mechanism proposed in our model, use of XEN602 added the question of why DMT-1 decreases movement of Tf.
2.6. References


McKie AT et al. (2000) A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation Molecular cell 5:299-309


Yoshikawa T, Pardridge WM (1992) Biotin delivery to brain with a covalent conjugate of avidin and a monoclonal antibody to the transferrin receptor The Journal of pharmacology and experimental therapeutics 263:897-903

Chapter 3

The role of sex and a common HFE gene variant in brain iron uptake

3.1. Abstract

HFE is an essential protein for regulating iron transport into cells. Two common mutations of the HFE gene, C282Y and H63D, result in loss of this regulation and are associated with accumulation of iron within the cell. The cellular iron accumulation can reach toxic levels; and in its most clinically aggressive form is known as hemochromatosis. HFE is a key iron management protein that is responsible for limiting cellular iron uptake. The mutated protein has been found increasingly in a range of neurodegenerative disorders. Additionally, recent evidence that the mutation is associated with elevated brain iron challenges the paradigm that the brain is protected by the blood-brain barrier (BBB). While much has been studied regarding the role of HFE in cellular iron uptake, it has remained unclear what role the protein plays in the transport of iron into the brain. Here, we investigated the regulation of iron transport into the brain using a mouse model with a mutation in the HFE protein. We demonstrated that 3-month old mice with the mouse homologue H67D/H67D mutation had significantly more brain iron than wild type mice, but that uptake of $^{59}$Fe was similar between the two genotypes. However, there were significant differences in the apparent rate of iron uptake between males and females regardless of genotype. These data suggest that brain iron status could be set during development while the brain is growing and that systemic iron changes or even mutations designed to favor iron uptake do not change brain iron levels.
3.2. Introduction

Iron is a vital micronutrient. The crucial nature of iron stems from its multifunctionality as a cofactor in various enzymatic reactions important for processes such as neurotransmitter synthesis, myelination, DNA synthesis and oxygen transport (Beard et al. 1996). Iron homeostasis is critical, however, due to its ability to easily transition between oxidation states through Fenton Chemistry. This reaction generates toxic hydroxyl radicals and reactive oxygen species, which can damage lipids, proteins, and DNA (Chevion 1988; Stohs and Bagchi 1995). The importance of brain iron imbalance is highlighted by the discovery of iron imbalances associated with various neurodegenerative diseases (Allen et al. 2001; Beard and Connor 2003; Connor et al. 2011; Connor et al. 1995; Forni et al. 2008; Oba et al. 1993; Zecca et al. 2004). Identifying the origin of brain iron imbalance has an additional challenge because the brain is protected by the blood-brain barrier (BBB), and regulation of iron transport at the BBB remains poorly understood. The BBB is the term given to the brain microvasculature, unique from that seen in most other organs due to its formation of tight junctions and complex mechanisms that regulate transport from the blood into the brain parenchyma (Abbott 2002; Abbott et al. 2010; Brightman and Reese 1969; Butt et al. 1990). A better understanding of the mechanisms by which iron is transported across the BBB may be crucial to determining the cause of brain iron imbalance in disease. Moreover, elucidation of the mechanisms of regulation of brain iron uptake and their regulation will be critical to optimizing intervention strategies for addressing behavioral and cognitive deficits that stem from developmental iron deficiency.

HFE is an iron management protein most often associated with the iron overload condition, hemochromatosis, but that has recently gained interest in the field of neurodegenerative disease (Meadowcroft et al. 2015; Nandar et al. 2013; Pulliam et al. 2003). The wild type HFE protein
functions on a cellular level to limit transferrin (Tf) binding to Tf Receptor (TfR)1 at the cell membrane (Feder et al. 1998; Lebron et al. 1999). HFE interacts with β2 microglobulin (β2M), a protein that directs HFE to the cell membrane where it can interact with TfR1 (Feder et al. 1998; Feder et al. 1997; Waheed et al. 1997). The interaction of HFE to TfR1 limits the receptor binding capacity to only one holo-Tf molecule (Feder et al. 1998; Lebron et al. 1999). The mutated form of HFE protein no longer decreases TfR1 binding capacity allowing for iron overloading to occur (Feder et al. 1998; Lebron et al. 1999). Two mutations in the HFE gene associated with the iron overload condition hereditary hemochromatosis (HH) were identified (Feder et al. 1996). Despite its increased association with HH, the C282Y gene variant is less prevalent in the population than the H63D gene variant.

In this study, we evaluated the H67D/H67D (mouse homologue to human H63D) HFE knock-in mouse. The HFE gene mutation is linked to the iron overload disease hemochromatosis and whether the brain accumulates iron in the presence of this mutation has been historically controversial, but the data clearly show the mutation is associated with increased brain iron and evidence is mounting that the prevalence of this mutation is elevated in numerous neurodegenerative diseases (Nandar and Connor 2011). Previous studies have confirmed the presence of HFE protein in the brain microvasculature and we have provided evidence that the presence of this mutation alters metabolism in a mouse model and in human brain (Ali-Rahmani et al. 2014; Meadowcroft et al. 2015; Nandar et al. 2013). The goal of the study was to evaluate changes in whole brain iron accumulation in a homozygous H67D/H67D mouse and to compare iron uptake patterns between wild type and H67D/H67D mice. Together, our previous data and the data presented here substantiate the concept of regulation of brain iron uptake at the level of the BBB (Ali-Rahmani et al. 2014; Nandar et al. 2013; Simpson et al. 2015).
3.3. Materials and Methods

3.3.1. Radiolabeling of Transferrin

$^{59}$Fe (40 uCi, Perkin Elmer) was complexed with 40 uL nitrilotriacetic acid (NTA), 0.5 uL 1 mg/mL ferrie chloride, and 2 uL 0.5 M sodium bicarbonate to form an $^{59}$Fe-NTA complex. Apo-Tf (1.2 mg, Sigma) was combined with the $^{59}$Fe-NTA complex and allowed to incubate for 10 minutes. Free iron was separated from the $^{59}$Fe-Tf using a Sephadex-G25 QuickSpin column as per the manufacturer’s instructions (Roche).

3.3.2. In vivo Iron Uptake Study

The H67D knock-in mice were used from our laboratory’s own colony. The development of these mice has been previously described (Tomatsu et al. 2003). Mice were genotyped to determine homozygosity, H67H/H67H or H67D/H67D, by our standard laboratory protocol (Nandar et al. 2013). Ten male and ten female H67H/H67H mice and ten male and ten female H67D/H67D mice received a single retro-orbital injection of 3.4 ug/gram body weight $^{59}$Fe-Tf. Injections were administered in the morning of a reverse 12-hour light/12-hour dark cycle. After 24 hours or 5 days, blood was collected and then the mice were transcardially perfused using 0.1 M PBS (pH 7.4). The mice were then decapitated and organs were collected and weighed immediately. Two mice were combined for each n = 1 to decrease radioactivity measurement error. For the brain, the cerebrum was removed from the cerebellum, the meninges were dissected, and the microvessels were subsequently isolated as described below. Radioactivity was then measured in all resulting brain fractions, blood, and liver on a Beckman Gamma 4000 (Beckman Coulter).
Protein in the microvessel isolates was measured by BCA assay as per the manufacturer’s instructions (Pierce). All procedures were conducted according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee.

### 3.3.3. Isolation of Microvessels

The brains were placed into a hand homogenizer unit containing 2 mL MVB Buffer (0.147 M NaCl, 0.4 mM KCl, 0.3 mM CaCl$_2$, 0.12 mM MgCl$_2$, 15 mM HEPES, 0.5% BSA, 5mM glucose). A sample of homogenized whole brain was taken to measure total brain radioactivity. Brain homogenate was transferred into a microcentrifuge tube and centrifuged at 1,000 x g for 10 minutes at 4°C. Supernatant was removed and a portion was used to measure radioactivity. The pellet was resuspended in 1 mL of 1.015 g/mL percoll. The resuspended pellet was layered on 3 mL of 1.05 g/mL percoll then centrifuged at 15,000 x g for 30 minutes at 4°C. The gradient was pierced by a needle and the microvessel-containing layer was collected. A myelin-containing fraction was also collected and radioactivity measured. The microvessel fraction was passed through a 100 um mesh filter. The remaining isolate was centrifuged at 1,000 x g for 10 minutes at 4°C for final microvessel recovery. The final microvessel pellet was then resuspended in RIPA buffer containing 1X protease inhibitor cocktail (Sigma) and homogenized for radioactivity and protein detection.

### 3.3.4. Measurement of Brain Iron

Five male and five female 3-month-old wild type and homozygous (H67D/H67D) H67D knock-in mice were transcardially perfused with 0.1 M PBS before sacrifice. Brains were harvested and
stored at -80°C. Frozen brain tissue was digested in 0.2% Nitric Acid at 60°C until digestion was complete (approximately 24 hours). The samples were then diluted 2-fold in TraceSELECT water (Fluka). Total brain iron concentrations (µg/g of tissue; wet weight) were measured by flame atomic absorption spectrometry according to standard protocol (PerkinElmer AAnalyst 800 Atomic Absorption Spectrometer).

3.3.5. Statistical Analyses

Prism (GraphPad Software) software was used for all statistical analyses and data graphing. Data are expressed as mean ± standard error of the mean. Statistical differences between experimental groups were determined using a two-way ANOVA and Bonferroni’s multiple comparisons test. A level of significance of p < 0.05 was used for all differences evaluated.

In vivo uptake study: All variables were initially summarized with frequencies and percentages for categorical variables and with means, medians, and standard deviations for continuous variables. The distribution of the main outcome, percentage total injectate, was checked using a histogram and normal probability plot. The distribution was determined not to follow normal population distribution, so nonparametric methods were employed for analysis instead. Comparisons using Wilcoxon Rank Sum tests were made between sexes within genotypes and between genotypes within sexes. These comparisons were made within each of the two time points and six sample types. Comparisons between time points within each sex, genotype, and sample combination were made using Wilcoxon Signed Rank tests. The change between time points was compared between genotypes within sexes or between sexes within genotype for each sample using Wilcoxon Rank Sum tests. To adjust for multiple comparisons, we applied the Bonferroni correction by multiplying the p-values by a factor of four to account for the four
comparisons made within each similar set of comparisons (sexes within genotypes and time points, genotypes within genotypes and time points, time points within sexes and genotypes). Medians and interquartile range were used to quantify differences between groups. All analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC).

3.4. Results

To determine if the brain iron uptake patterns could be altered by a systemic mutation that favors iron loading in organs, we examined mice carrying the H67D mutation of the HFE gene. Indeed, these mice have a significant increase in brain iron at 3 months of age compared to wild type (Figure 3-1). Liver $^{59}$Fe uptake was measured as an organ associated with elevated iron in the HFE mutants (Figure 3-2). $^{59}$Fe uptake was increased in the H67D/H67D female mice compared to wild type but not in males at 24 hours post-injection (Figure 3-2). Five days after injection, female mice had elevated $^{59}$Fe compared to males but there was no genotype effect (Figure 3-2).
Figure 3-1: **Total brain iron.** Total brain iron was measured in H67D/H67D and wild type mice by atomic absorption. There was significantly more iron in the brain of H67D/H67D mice when compared to their wild type counterparts. Iron in H67D/H67D males was ~37% higher than wild type males while iron in H67D/H67D females was ~61% higher than wild type females. n = 5/group. ***p < 0.001, ****p < 0.0001.
Figure 3-2: $^{59}$Fe accumulation in the livers of wild type and H67D/H67D mice. A) At 24 hours after injection, livers were collected, weighed, and radioactivity was measured. H67D/H67D females exhibited significantly more $^{59}$Fe retention in the liver, but no other changes were observed. B) At 5 days after injection, there were no significant differences in $^{59}$Fe liver accumulation across genotypes, but there was significantly more $^{59}$Fe retained in the liver of female mice when compared to their male counterparts. *p < 0.05.

Accumulation of $^{59}$Fe was analyzed in whole brain, a cortical brain fraction, a myelin-containing fraction, and a microvessel-containing fraction (Figures 3-3 – 3-6). There were no significant genotype differences of $^{59}$Fe accumulation observed at 24 hours in any of the brain fractions. Of note, however, there was a significant sex effect observed at 5 days after injection. Females have higher levels of $^{59}$Fe in the female whole brain, cortical, and myelin-containing fractions. Moreover, the wild type males appeared to lose iron over the 5-day period but the other groups maintained similar amounts of iron (Figure 3-3C). The HFE mutant mice had 81% less iron in the microvasculature at 24 hours post injection but this difference did not reach statistical significance. After 5 days, male wild type mice still had 42% higher iron than the mutant mice.
but this finding was still not statistically different. Of note, the amount of iron in the microvasculature was unchanged over 5 days in each of the groups indicating a stable level of iron in the BBB cells (Figure 3-6C). The loss of iron over 5 days in the wild type male appears to be driven by a failure of the white matter (myelin fraction) to retain iron (Figure 3-5C).
Figure 3-3: $^{59}$Fe accumulation in whole brain. A) At 24 hours after injection with $^{59}$Fe-Tf, brains were homogenized and radioactivity was measured. No significant sex or genotype effects were observed. B) At 5 days after injection with $^{59}$Fe-Tf, brains were homogenized and radioactivity was measured. There was significantly more $^{59}$Fe present in the brains of female mice than in the brains of male mice for both genotypes studied. C) Changes in $^{59}$Fe between the 24-hour and 5-day time points was calculated. Wild type females exhibited significantly less change than the wild type males, which had reduced $^{59}$Fe. *p < 0.05.
Figure 3-4: $^{59}$Fe accumulation in an isolated cortical brain fraction. A) After 24 hours, no significant sex or genotype effects were observed. B) Five days after injection, females for both genotypes exhibited significantly more cortical $^{59}$Fe accumulation when compared to males within the same genotype. C) There were no significant differences in the change seen in cortical brain levels of $^{59}$Fe. *p < 0.05.
Figure 3-5: $^{59}$Fe accumulation in a myelin-containing brain fraction. A) No significant sex or genotype effects were seen at 24 hours after injection. B) At 5 days after injection, however, there was significantly more $^{59}$Fe in the myelin-containing brain fraction of females when compared to males within the same genotype. C) The change in iron associated with the myelin-containing fraction was calculated. The wild type males exhibited a greater reduction in $^{59}$Fe over the 5-day time period than wild type females or H67D/H67D males. *p < 0.05.
Figure 3-6: $^{59}$Fe accumulation in the brain microvasculature. A) Within 24 hours after injection, the brain microvasculature accumulated measurable levels of $^{59}$Fe. B) After 5 days, the brain microvasculature retained $^{59}$Fe. C) The microvasculature exhibited almost no change in $^{59}$Fe over the 5-day time course studied here, indicating its ability to retain iron and function as an iron reservoir.
3.5. Discussion

The existing paradigm regarding the HFE mutation is that there is no increase in brain iron accumulation with this mutation because of the BBB. Originally, however, it was reported that patients with hemochromatosis showed increased brain iron staining in areas that were protected by the BBB and those areas not protected by the BBB. (Cammermyer 1947; Sheldon 1935). Moreover, more recent papers using neuroimaging have reported an increase in brain iron in individuals with the HFE gene variant (Meadowcroft et al. 2015) and animal studies from our own laboratory with the knock-in model of the HFE/H63D gene variant (Nandar et al. 2013) report brain iron accumulation. The focus of this study was to determine how brain iron uptake mechanisms are affected by the HFE gene variant. We found that at 3 months of age, the mutant mice had increased levels of total brain iron but no significant differences in the rate of \(^59\)Fe uptake over 24 hours or 5 days in the brain when compared to their wild type counterparts. These data are perhaps the strongest support for the concept we introduced in our previous study (Simpson et al. 2015) that signaling regarding brain iron status comes from the brain and that even a genetic mutation that is geared towards iron overload does not result in elevated iron uptake.

The HFE mutation (H67D) under investigation in this study is the most common gene variant in Caucasians (Merryweather-Clarke et al. 1997) and the impact of this gene variant is seen in amyotrophic lateral sclerosis, Alzheimer’s disease and Parkinson’s disease (Nandar and Connor 2011). These diseases all exhibit iron dyshomeostasis and disruptions in cholesterol metabolism (Ali-Rahmani et al. 2014; Nandar and Connor 2011; Zecca et al. 2004). The importance of the HFE mouse model, is that it enables us to determine if and how brain iron uptake is regulated using a genetic model favoring iron overload. That the brains from the mutant had elevated iron
levels but that brain iron uptake was not different between the mutant and wild type mice indicates there is a set point for brain iron status that is set earlier in development.

Indeed, the similarity in iron uptake in the wild type and mutant also indicates that the signals coming from the brain were comparable despite the 33% increase in brain iron content in the mutant mice; there was not a reduction in uptake suggesting the two models had reached a brain iron set point that was being maintained. There still may be an impact of the HFE gene variant on handling of iron within the endothelial cells of the BBB because of the 42% (females) and 81% (males) decrease in iron in the endothelial cells associated with the HFE mutation but this difference did not reach statistical significance in this study and will be further evaluated in future studies.

The observation of iron in the microvasculature represents a key finding of our study. Not only did our data demonstrate that there was iron accumulation in the microvessels after the initial 24 hours post-injection but $^{59}$Fe was also present 5 days after the injection. These data are strong support for our concept that the BBB serves as an iron reservoir for the brain parenchyma (Simpson et al. 2015) and are consistent with our finding in this study and previous in vivo studies that ferritin is expressed in the brain microvasculature where iron can be stored (Burdo et al. 2004).

Another key observation in this study was the sex-dependent differences in $^{59}$Fe accumulation in the brain. Our study replicates findings by Tomatsu, et al. in which the female but not male H67D/H67D mice accumulate more liver iron when compared to wild type (Tomatsu et al. 2003) and extends the sex-dependent differences in iron uptake to include iron accumulation in the brain. To date, studies that evaluated sex differences in brain iron have revealed mixed results.
While there are studies that have shown elevated ferritin and iron in the male brain compared to female (Bartzokis et al. 2007; Persson et al. 2015), there was also another study that could not replicate these findings (Xu et al. 2008). The higher levels of ferritin described in the male brain may suggest that more of the iron taken up into the male brain is stored whereas the iron taken up into the female brain is utilized immediately.

An explanation for the sex-dependent differences may be in the role for iron in brain functions such as neurotransmitter synthesis and myelination. The sex differences in brain iron uptake are consistent with reports that females have higher rates of dopamine synthesis and release when compared to males (Andersen et al. 1997; Bazzett and Becker 1994; Becker 1990; Castner et al. 1993; McDermott et al. 1994; Munro et al. 2006). A well-established connection between iron status and dopamine has been described (Beard et al. 2003; Bianco et al. 2008; Erikson et al. 2000; Erikson et al. 2001; Youdim et al. 1989; Youdim 1990; Zaleska et al. 1989). This impact of iron on dopamine biology is in part due to its role as a cofactor for tyrosine hydroxylase, the enzyme responsible for generating the direct dopamine precursor L-DOPA (Fitzpatrick 1989; Goodwill et al. 1997). Additionally, the sex differences are consistent with reports of increased myelination within the female adult brain (Bayless and Daniel 2015; Yang et al. 2008). A relationship between iron and myelination is also well-established (Connor and Menzies 1996; Oski et al. 1983; Todorich et al. 2009). Thus, sex differences in iron uptake may be the result of sex-dependent brain iron requirements. These findings may have direct implication for clinical studies as they suggest females may be at greater risk for decreased cognitive and motor function associated with iron deficiency than males.

The impact of the HFE gene variant on brain iron accumulation appears to be age dependent. We demonstrated in this study there is more iron in the brain of the mutant animals at 3 months of age
and previously reported an increase in brain iron content at 6 months of age in the mutant but that the difference in iron levels no longer were present by 12 months of age (Nandar et al. 2013). The total brain iron data suggests there is a compensatory response that limits iron accumulation in the mutant but not in the wild type as the latter continues to accumulate iron between 6 and 12 months. After 12 months of age, there is an apparent breakdown of the compensatory response and the mutant animals once again accumulate significant amounts of iron and show significant behavioral and cognitive impairment (Ali-Rahmani et al. 2014). Our data on brain iron uptake suggest that the compensatory response to limit iron uptake by the mutant has occurred by 3 months of age. As we noted, there was 81% less iron in the microvasculature at 24 hours in the mutants compared to wild type.

This study provides further evidence for regulation of brain iron uptake. The uptake data from the mutant and wild type mice argue that the brain reaches a set point for iron status during development that is maintained independent of the iron content of the brain. Iron uptake is not increased to maintain the elevated brain iron status nor is it decreased in response to elevated brain iron. Presumably, this set point is established during the period of rapid brain growth because the strongest correlation for iron uptake by the body is growth (Dallman 1992). Once the growth of the brain is established, the iron status is maintained. The corollary of these data would suggest that it could be difficult to provide supplements to enhance brain function once a set point has been reached. This is evidenced in the case of brain iron deficiency in which data show that cognitive deficits persist into adulthood despite recovery of systemic iron levels (Algarin et al. 2013; Corapci et al. 2006; Lukowski et al. 2010; Roncagliolo et al. 1998). Our data show the maintenance of normal iron status is different for males and females and the amount of iron uptake for normal brain function are different. This observation could clearly impact dietary
strategies for maintaining a healthy population. Clearly, the HFE model provides considerable
opportunity to explore the regulatory mechanism of brain iron uptake.
3.6. References

Bazzett TJ, Becker JB (1994) Sex differences in the rapid and acute effects of estrogen on striatal D2 dopamine receptor binding Brain research 637:163-172 doi:http://dx.doi.org/10.1016/0006-8993(94)91229-7


Feder JN et al. (1996) A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis Nature genetics 13:399-408 doi:10.1038/ng0896-399

Feder JN et al. (1998) The hemochromatosis gene product complexes with the transferrin receptor and lowers its affinity for ligand binding Proceedings of the National Academy of Sciences of the United States of America 95:1472-1477


Fitzpatrick PF (1989) The metal requirement of rat tyrosine hydroxylase Biochemical and biophysical research communications 161:211-215


Munro CA et al. (2006) Sex Differences in Striatal Dopamine Release in Healthy Adults. Biological Psychiatry 59:966-974 doi:http://dx.doi.org/10.1016/j.biopsych.2006.01.008


Chapter 4

Evaluation of Brain Iron Uptake using Cerebrospinal Fluid from Restless Legs Syndrome patients

4.1. Abstract

Restless Legs Syndrome (RLS) is a disease characterized by an urge to move the legs. The brain of RLS patients has been characterized with iron deficiency that is not necessarily reflected systemically. Currently, the main predictive measure for treatment of RLS with intravenous iron is serum ferritin. Here, we sought to determine the impact of cerebrospinal fluid (CSF) from RLS patients on brain iron uptake and correlate uptake with systemic measures of iron status. We show that cerebrospinal fluid impacts the transport across and the release of iron from endothelial cells correlates with the systemic hemoglobin status of these individuals. Specifically, lower hemoglobin samples were associated with significantly less transport when compared to higher hemoglobin samples. Importantly, serum ferritin had no association to iron transport across the in vitro blood-brain barrier (BBB) model but there was a strong correlation to hemoglobin levels. The data suggest that the RLS brain does not release signals to increase brain iron uptake in response to the brain iron deficiency. Systemic iron status also may have little predictive value on movement of iron into the brain. We explore the possibility that the correlation to hemoglobin may be a key finding that instead implicates hypoxia in the pathogenesis of RLS.

4.2. Introduction

Restless Legs Syndrome (RLS) is a neurological disorder that is characterized by a strong urge to move the legs. Currently, RLS is treated primarily by dopamine agonists and iron supplementation (Akpinar 1982; Allen et al. 2013; Allen and Earley 1996; Benes et al. 1999; Cho
et al. 2013; Oski et al. 1983; Thorpy 2005; Trenkwalder et al. 1995; Walters et al. 2004; Wang et al. 2009). The use of dopamine agonists, however, only treats the symptoms and often results in augmentation, the term given to returning symptoms after dopamine agonist treatment (Allen and Earley 1996). Additionally, there is strong and consistent data that RLS is associated with insufficient brain iron (Allen et al. 2001; Clardy et al. 2006; Connor et al. 2011; Earley et al. 2014; Earley et al. 2000; Mizuno et al. 2005) We have previously shown alterations in cerebrospinal fluid (CSF) of iron, Tf and ferritin levels in the CSF from RLS patients (Connor et al. 2011) and we have demonstrated that CSF from iron deficient monkeys could increase iron release in our BBB model (Simpson et al. 2015). Notably, the RLS brain mimics the changes observed in brain iron insufficiency, including decreased myelination (Allen et al. 2001; Chang et al. 2014; Connor et al. 2009; Earley et al. 2014; Earley et al. 2006). Moreover, brain iron insufficiency negatively impacts the dopaminergic system causing decreased dopamine reuptake, transport, and receptor expression (Bianco et al. 2008; Erikson et al. 2000; Erikson et al. 2001; Jellen et al. 2013; Unger et al. 2008; Zaleska et al. 1989). The mechanism by which the brain in RLS could become iron deficient could provide a model for interrogation of adult brain iron deficiency and regulation of brain iron uptake.

The brain is protected by the blood-brain barrier (BBB). The term BBB is given to the brain microvasculature, characterized by the formation of tight junctions between the endothelial cells (Abbott 2002; Abbott et al. 2010; Brightman and Reese 1969; Butt et al. 1990) and functions to tightly regulate movement from the blood into the brain parenchyma. Currently the existing paradigm for iron transport across the BBB has been expanded from a simple conduit to include an endocytic mechanism by which the endothelial cells can obtain iron and also release iron into the parenchyma on demand from signals within the brain (Simpson et al. 2015). The ability to regulate iron release from the BBB suggests that the brain secretes signals to the BBB regarding
its iron status. Thus the insufficient levels of brain iron in RLS raises two questions: 1) Does the RLS brain recognize the altered brain iron status and secrete appropriate signals? 2) Does the BBB respond properly to signals of altered brain iron status?

To better understand the cause of altered brain iron status in patients with RLS we determined the impact of CSF from brains of RLS and control patients on brain iron transport in a cell culture model of the BBB. Furthermore, because serum ferritin levels are used clinically as a decision point for treating with intravenous iron (Allen et al. 2013; Wang et al. 2009), we also interrogated the relationship between serum ferritin and transport in the BBB model.

4.3. Materials and Methods

4.3.1. Radiolabeling of Transferrin

$^{59}\text{Fe}$ (40 uCi, Perkin Elmer) was complexed in a solution of 40 uL nitrilotriacetic acid (NTA), 0.5 uL 1 mg/mL ferric chloride, and 2 uL 0.5 M sodium bicarbonate to form an $^{59}\text{Fe}$-NTA complex. The $^{59}\text{Fe}$-NTA complex was incubated with apo-Tf (1.2 mg, Sigma) 10 minutes. Free iron was removed from the complexed iron using a Sephadex-G25 QuickSpin column was used as per the manufacturer’s instructions (Roche).

4.3.2. Cell Culture

Bovine brain microvascular endothelial cells (BBMVEC) were cultured in complete growth medium (Cell Applications, Inc.). For functional assays, BBMVECs were grown to confluence on
Costar transwell 0.4 µm porous filters (Corning). Upon reaching confluence, the BBMVECs were gently washed three times with 1X DPBS (Corning). BBMVECs were then maintained in serum free media containing 138 nM hydrocortisone for 72 hours. Hydrocortisone supplementation has been shown to induce the formation of tight junctions by upregulating associated proteins including occludin and ZO-1 (Antonetti et al. 2002; Kurzen et al. 2002).

4.3.3. Release Assay

BBMVECs in the transwell setup were loaded overnight with 10 uCi/well of $^{59}$Fe-NTA complex, generated as previously described (Bali et al. 1991). After the overnight loading, wells were washed three times with 1X DPBS (Corning) to remove iron remaining in the culture medium. Serum-free medium containing 70 kDa RITC-Dextran (Sigma Aldrich) was added to the apical chamber to function as a measure of barrier integrity in this model as this size of dextran has been shown to not cross the BBB freely. CSF from female patients with RLS or from healthy controls was implemented in the basal chamber of our model to demonstrate the impact that CSF from a diseased brain has on iron release from the BBB (Table 4-1). Samples were received from both Johns Hopkins University and Emory University. Of note, the samples from Johns Hopkins University were pooled because of low sample volume; two patient samples were pooled to represent n = 1. Data were collected by taking 50 uL aliquots from the apical chamber at 0 and 6 hours and the basal chamber at 0, 2, 4, and 6 hours. Fluorescence of the samples was measured on a SpectraMax Gemini EM plate reader (Molecular Devices) and $^{59}$Fe was then measured on a Beckman Gamma 4000 (Beckman Coulter).
Table 4-1: Patient demographics for RLS CSF *in vitro* BBB assays.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>RLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>48 (36, 62)</td>
<td>48.5 (37, 60)</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>13.2 (12, 14.125)</td>
<td>13.25 (12.925, 14.1)</td>
</tr>
<tr>
<td>Serum Ferritin (ng/mL)</td>
<td>597.0 (568.5-651.4)</td>
<td>574.1 (523.5-666.5)</td>
</tr>
</tbody>
</table>

Data are represented as median (QL-QH)
n=18 for the Control group
n=16 for the RLS group

4.3.4. Transport Assay

Tf flux across the *in vitro* BBB was measured by applying \(^{59}\text{Fe-AlexaFluor 488-Tf}\) to the apical chamber of our transwell model. Fresh media containing the \(^{59}\text{Fe-AlexaFluor 488-Tf}\) and 70 kDa RITC-Dextran was added to the apical chamber at the beginning of the experiment. For the transport study, CSF from female patients with RLS or from healthy controls was used in the basal chamber of our model. For all conditions, media aliquots were taken from the apical chamber at the 0-hour time point and the end point. Samples from the basal chamber were taken at 2-hour intervals over 10 hours. Fluorescence was measured in all samples on a SpectraMax Gemini EM plate reader (Molecular Devices) and \(^{59}\text{Fe}\) was measured on a Beckman Gamma 4000 (Beckman Coulter). To evaluate the ratio of protein-bound versus free iron, media was passed through a Sephadex G-25 Quick Spin column as per the manufacturer’s instructions (Roche).
4.3.5. Statistical Analyses

Prism (GraphPad Software) software was used for all statistical analyses and data graphing. Data from three independent replications were pooled and are expressed as median ± interquartile range. Statistical differences between experimental groups were determined using two-way ANOVA and Bonferroni’s multiple comparisons test. A level of significance of $p < 0.05$ was used for all differences evaluated.

4.4. Results

To examine a neurological disorder associated with brain iron deficiency on brain iron transport and release from endothelial cells, we obtained CSF from patients with RLS. Previously we have shown that CSF from infant monkeys that were iron deficient was associated with increased iron release from the BBB cells in culture (Simpson et al. 2015). Iron transport was not significantly changed when the endothelial cells were exposed to CSF from control or RLS subjects (Figure 4-1A). For the iron release studies, we only had enough CSF to examine the patient samples received from Emory University. CSF from both RLS and control subjects induced similar amounts of release from the $^{59}$Fe-loaded endothelium (Figure 4-1B).
Figure 4-1: Effect of CSF from healthy and RLS patients on iron transport and release in an *in vitro* BBB model. A) Iron flux across the *in vitro* BBB model was not significantly different between the control and RLS groups. B) CSF from RLS patients had no significant impact on iron release from the cultured BBB model. *p < 0.05.
In our previous study on infant monkeys, we demonstrated a correlation between hemoglobin levels and iron release from endothelial cells (Simpson et al. 2015). Therefore, we examined both $^{59}\text{Fe}$ transport and $^{59}\text{Fe}$ release in relation to hemoglobin. We found a positive correlation between hemoglobin and both transport (R-square = 0.33, p = 0.004, Figure 4-2A) and release (R-square = 0.63, p = 0.05, Figure 4-2B) of $^{59}\text{Fe}$. Because serum ferritin is used as a clinical indicator for treatment in RLS (Allen et al. 2013; Wang et al. 2009), we examined transport of $^{59}\text{Fe}$ in relation to serum ferritin levels but found no correlation (Figure 4-2C).
Figure 4-2: Correlation of iron transport and release to systemic iron measures. A) Transport exhibited a weak positive correlation to systemic Hgb levels (R-square = 0.33, p = 0.004). B) There was a positive correlation between systemic Hgb levels and iron released from the in vitro BBB model (R-square = 0.63, p = 0.05). C) There was no significant correlation between iron transport and serum ferritin (R-square = 0.05, p = 0.35). Grey squares represent control patients and black circles represent RLS patients.
4.5. Discussion

In this study, the question of whether the CSF contains signals to influence iron release was examined in a model of human neurological disease associated with iron deficiency; namely RLS. CSF from RLS patients was not associated with significant changes in iron transport into the brain. There was a significant, positive correlation between hemoglobin levels and iron transport that was not dependent on the source of the CSF. Because serum ferritin levels are used clinically as a decision point for treating with intravenous (IV) iron (Allen et al. 2013; Wang et al. 2009), we interrogated the relationship between serum ferritin and transport in the BBB model and found no correlation. These data suggest that hemoglobin levels may be useful in predicting responders to IV iron in the RLS patient population.

The correlation between iron transport and iron release and hemoglobin levels introduces an intriguing possible interpretation. A key point to note here is that despite hemoglobin’s common association with systemic iron levels, the protein’s primary function is in oxygen transport. Early developmental studies of iron deficiency proposed the concept of a hierarchy in which tissues forgo their iron needs to maintain erythropoiesis and oxygen status (Georgieff et al. 1989; Guiang et al. 1997; Veng-Pedersen et al. 1993; Zamora et al. 2016). For example, in a study on phlebotomized lambs, iron was preferentially directed to red blood cells (RBCs) after liver iron dropped below a critical threshold level (Guiang et al. 1997). Furthermore, newborn infants exposed to hypoxemia in utero demonstrated decreased serum and tissue-associated iron (Georgieff et al. 1989); suggesting the iron was diverted to RBCs to support hemoglobin synthesis and to address depressed oxygen saturation (Georgieff et al. 1989). In a direct experiment to assess the brain iron status, a recent study demonstrated in phlebotomized lambs that brain iron decreases corresponded to increased RBC total iron content suggesting that iron
was being preferentially distributed to the RBCs (Zamora et al. 2016). Similar results were found in iron-deficient monkeys in which proteins involved in processes such as synapse formation remained altered after systemic iron parameters were resolved with iron-sufficient diet (Geguchadze et al. 2008; Patton et al. 2012). Our data revealing the correlation between iron transport in the BBB model and hemoglobin is consistent with the concept that iron was prioritized to erythrogenesis to provide increased oxygen transport. This interpretation is consistent with our reports that the brains of RLS patients show evidence of activation of hypoxic pathways (Patton et al. 2011). In general, it appears that the brain will tolerate some degree of iron deficiency, even that which impacts myelin formation and dopaminergic function that are impaired in RLS, to maintain sufficient levels of oxygen saturation and hence oxygen delivery by the RBCs.

Key to understanding our findings in this study is identification of the signaling mechanism between the CSF and endothelial cells for iron transport. We have shown that apo-Tf (iron poor transferrin) will increase release of iron from endothelial cells but not transport across them. In addition, CSF from iron deficient monkeys resulted in increased release of iron from the BBB model compared to iron sufficient monkeys. In the monkey study, we examined iron release from endothelial cells rather than transport of iron. It is possible that transport would decline with increased competition for serum iron when hemoglobin is low while release of iron already stored in the endothelial cells would be increased. However, there was no difference in release of iron between control and RLS CSF, although the sample size was small. There was a positive correlation between serum hemoglobin and iron release in this study; unlike the negative correlation we found in the study with monkey CSF. At this time, explanations for the differences between the monkey and human data could involve species or age differences (the monkeys were infants), the correlation between iron uptake and release and hemoglobin is
entirely consistent with our model of regulation of iron transport at the level of the BBB and that the BBB serves as a reservoir of iron for the brain (Simpson et al. 2015). However, this interpretation implies there is a signal in the CSF of the RLS patients or even the control subjects with lower hemoglobin that would inform the endothelial cells of the BBB to decrease iron transport and release of iron. This could be a key concept in the mechanism underlying the insufficient amounts of iron in the brain in RLS and will be explored in future studies.
4.6. References


Guiang SF, 3rd, Georgieff MK, Lambert DJ, Schmidt RL, Widness JA (1997) Intravenous iron supplementation effect on tissue iron and hemoproteins in chronically phlebotomized lambs The American journal of physiology 273:R2124-2131


erythropoietin in the sheep fetus; evidence of threshold response in spontaneous hypoxemia Journal of pharmaceutical sciences 82:804-807
Zamora TG, Guiang SF, 3rd, Widness JA, Georgieff MK (2016) Iron is prioritized to red blood cells over the brain in phlebotomized anemic newborn lambs Pediatric research 79:922-928 doi:10.1038/pr.2016.20
Chapter 5

Regulation of Brain Iron Status – Summary and Implications

5.1. Summary of Main Findings of Dissertation

The data presented in this dissertation indicate that signals from the brain regulate brain iron uptake at the blood-brain barrier (BBB). Although seemingly intuitive, it was only recently that the historical paradigm that the BBB was a passive conduit allowing the unregulated transcytosis of transferrin was challenged by our laboratory (Simpson et al. 2015). The release of iron from an in vitro model of the BBB was induced by the presence of iron-poor transferrin (apo-Tf), which represents a potential physiological iron chelator, and the pharmacological iron chelator, deferoxamine (DFO). In Restless Legs Syndrome (RLS), used here as a human model of brain iron deficiency, cerebrospinal fluid (CSF) did not significantly impact release or transport of iron. Moreover, despite significant increases in total brain iron at 3 months of age, mice with a genetic predisposition to iron overload had a similar apparent rate of iron accumulation compared to wild type animals. Previously our laboratory had shown that CSF from monkeys that were iron deficient increased iron release from the BBB model. Thus, taken together the data from the RLS CSF and the animal data suggest that the adult brain does not identify as having an iron imbalance; either increased or decreased. The data argue for the establishment of a set point for iron uptake. The data presented in this thesis introduce the concepts that brain iron concentration is determined during early development and that this level is not readily altered in the adult brain. This latter statement provokes the question of how feasible it is to attempt to therapeutically alter iron dyshomeostasis in the adult brain. Our release studies with apo-Tf may indicate that the ability to address iron dyshomeostasis on a local level in brain regions where changes in iron utilization may be reflected in local accumulations of apo-Tf, but general deficiencies in brain
iron status that may be reflected in CSF (an overall indicator of brain extracellular fluid) may not be reversible. Overall, our data suggest that global brain iron status is set during development and the potential corrective mechanisms are regional and specific to the adult brain.

The distribution of iron seen in the adult brain appears to occur by postnatal day 30 (Connor and Fine 1987), although brain iron distributions change during development. During the early stages of brain growth, brain iron is most concentrated in the globus pallidus, caudate nucleus, and putamen (Aoki et al. 1989; Dwork et al. 1990). However, regions such as the substantia nigra and red nucleus do not exhibit higher regional brain iron until later in development (Dwork et al. 1990). The question remains – is this a redistribution of iron or an example of regional differences in uptake? The possible mechanism underlying regional iron variation may be explained by the metabolic iron needs of each specific brain region. For example, as discussed later in this chapter, the iron-rich substantia nigra is a key site for the iron-dependent dopamine synthesis mechanism.

5.2. When Are There Signals to the BBB About Brain Iron Status?

Much of what is known about the role of iron in brain maturation has been established by identifying the detrimental effects of iron deficiency on the brain. The brain is distinguished from other organs in the body on the basis of its high metabolic activity. In fact, the newborn brain accounts for nearly 80% of the body’s resting metabolic rate (Kuzawa and Kuzawa 2010). The need for iron is, therefore, high to support iron-dependent brain metabolic processes such as myelogenesis, neurotransmitter synthesis, and synaptogenesis (Beard et al. 1996). During development, brain iron accumulates most rapidly in the early postnatal stage. The hippocampus and striatum are the regions undergoing most development at this time but no studies have
addressed whether or not iron is selectively targeted to these regions. (Connor and Fine 1987; Connor and Menzies 1996; Georgieff 2008; Roncagliolo et al. 1998). The increases in iron coincide with the appearance of myelin basic protein expression and increases in transferrin expression (from developing oligodendrocytes), implicating myelin as a driving force for iron acquisition by the developing brain (Connor and Menzies 1996; Moos and Morgan 2002). The regional dependency within the brain on iron for metabolic needs is exemplified in a study by Dallman who demonstrated losses of cytochrome C during iron deficiency (Dallman 1986), which was later shown to be primarily associated with the hippocampus (de Deungria et al. 2000). Decreased cytochrome C and cytochrome C oxidase in the presence of iron deficiency indicate decreased metabolic activity in the brain, which is essential for neurogenesis and is associated with delayed maturation of key brain regions such as the hippocampus (Callahan et al. 2013; Dallman 1986; de Deungria et al. 2000; Rao et al. 2013). Also during the early postnatal stage, the dopaminergic system is rapidly developing. Studies of iron deficiency have demonstrated decreased reuptake of dopamine, which results in increased levels of extracellular dopamine (Nelson et al. 1997), as well as decreased dopamine receptors (Erikson et al. 2001). A key remaining question is whether iron uptake shifts among different brain regions relative to their level of activity during development. If brain iron uptake shifts, then we would expect to see temporal-dependent effects of iron deficiency on specific brain regions (i.e. hippocampus, nigrostriatal pathway, myelin). Given that myelin production is highly metabolically active and global within the brain, the driving force for brain iron uptake could still be myelination.

There is considerable evidence that iron deficiency during early development may persist and be detrimental even when the systemic iron deficiency is corrected. Rats that were fed an iron deficient diet from postnatal day 10 until either day 28 or day 48 continued to show decreased brain iron concentrations even after 45 days of iron supplementation, which suggests that global
brain iron insufficiency is not correctable (Dallman et al. 1975). Furthermore, ferritin levels in the brain remained depressed by 33-42% (Dallman et al. 1975) indicating less brain iron was present. In human studies as well, the detrimental impact of brain iron deficiency during early maturation has been demonstrated. For example, iron-deficient infants identified at age 6, 12, or 18 months were given an iron supplement for 6 months and later evaluated at age 10 (Algarin et al. 2013). The previously iron-deficient children exhibited both slower reaction times and decreased inhibitory control, consistent with dopaminergic system dysregulation (Algarin et al. 2013). Moreover, in a study that introduced iron therapy to iron-deficient 12-23 month old toddlers, similar results were observed, but expanded to include poor planning as well as impaired hippocampus-based recognition memory (Lukowski et al. 2010). These studies are supported by many other similar studies in which children received early childhood supplementation and failed to fully recover to normal behavioral measures (Christian et al. 2011; Corapci et al. 2006; Murray-Kolb et al. 2012) Together, these studies suggest that the cognitive effects from iron deficiency during infancy cannot be reversed by later iron supplementation. Our studies showed that in both RLS, which is reportedly associated with brain iron deficiency (Allen et al. 2001; Clardy et al. 2006; Connor et al. 2011; Earley et al. 2014; Earley et al. 2000; Mizuno et al. 2005), and the H67D HFE mutant animals that have brain iron overload by 3 months, the signal(s) to induce or decrease iron movement across the BBB were not present. Based on these data, it seems that once the brain reaches a fixed point, iron supplementation will no longer be effective to address global iron imbalances. Therefore, to prevent the detriments of iron deficiency on brain development, the iron deficiency must be corrected before the set point is reached. This set point is likely reached after a critical period of brain iron requirement (Fox et al. 2010; Knudsen 2004). Presumably, the critical period happens in the very early postnatal timeframe because studies in rodents have demonstrated that iron repletion initiated at postnatal day 21 still resulted in abnormal behavior, suggesting that the irreversible change has already occurred (Beard et al.
Interestingly, this time period coincides with myelogenesis and the development of the dopaminergic system (Connor and Menzies 1996; Crowe and Morgan 1992; Tarazi and Baldessarini 2000).

As evidence that supplementation to improve brain iron status can work at some time-point, there are studies that evaluated the role of maternal iron supplementation on brain growth of the offspring as well as early infancy supplementation. Studies demonstrate that when mothers are prophylactically administered iron supplementation, the offspring had higher birth weights and, in an area where prevalence of iron deficiency is high, positive effects on memory, inhibitory control, and fine motor function were observed (Christian et al. 2010; Cogswell et al. 2003). In one study where term infants received iron supplementation, children had higher visual acuity and psychomotor developmental indexes, suggesting that supplementation may still be beneficial in the immediate postnatal time period (Friel et al. 2003).

Although there is significant literature on supplementation, there are also strong sentiments about whether or not infants should be supplemented with iron (Berglund et al. 2010; Friel et al. 2003; Furman 2011; Hare et al. 2015; Hernell and Lonnerdal 2011; Lozoff et al. 2012; Sachdev et al. 2005; Schanler et al. 2011; Singhal et al. 2000). This is due to studies suggesting that supplementation of iron-sufficient infants may be harmful to develop, causing decreased growth as an example (Dewey et al. 2002; Idjradinata et al. 1994; Lozoff et al. 2012; Majumdar et al. 2003). Our study with the H67D mutant animals indicate that more iron than normal can accumulate in the brain in association with a genetic modification. Iron can also accumulate in the brain when increased in the diet (Kaur et al. 2007). The authors fed mice an iron-fortified diet by oral gavage during postnatal days 10-17 and then measured both brain iron and the susceptibility to neurodegeneration. The brains of the mice fed an iron-fortified diet had increased
brain iron at 2 months of age (Kaur et al. 2007). Furthermore, the mice on the fortified diet were found to be more susceptible to toxic insult later in life. The authors exposed the mice to a toxin that induces Parkinsonian-like symptoms and found that the mice fed the fortified diet during early development had reduced dopamine in the striatum as well as decreased tyrosine hydroxylase positive neurons, both of which are characteristics of Parkinson’s Disease (Kaur et al. 2007). Much like the iron deficiency studies, this study demonstrates that the effects from iron fortification persist into adulthood despite normalization of iron intake. Together, the data emphasizes the need to identify the critical period of brain iron uptake so as to better address questions of supplementation.

In our H67D mutant animal study, we saw that by 3 months of age, the mutant mice were taking up iron at a rate comparable to the wild type mice, suggesting that the rate of brain iron uptake has leveled off. To address the timing at which brain iron uptake reaches its set point, several questions need to be answered. Firstly, the total iron in brains of the mice needs to be identified at birth and several early postnatal time points – based on studies of maternal iron supplementation, it may be possible that the H67D mice are already born with elevated levels of brain iron. Secondly, performing an uptake study like the one described in Chapter 3 at the early postnatal time points could help to determine the point at which the brain shifts to an iron uptake maintenance level. This time point would be identifiable as the time at which the H67D mice were taking up equal amounts of $^{59}$Fe as compared to the wild type mice. Furthermore, we have recently identified ferritin as a potential source of iron for oligodendrocytes (Todorich et al. 2011). Therefore, ferritin must also be considered as a potential source of brain iron and so studies evaluating both transferrin-bound iron (TBI) and ferritin-bound iron may be necessary to generate a complete profile of developmental brain iron uptake.
Identifying the iron management protein profile of the vasculature throughout development will be imperative to interpreting the uptake studies. In our H67D animal study, we saw similar rates of iron uptake between the mutant and wild type animals at 3 months of age. Because mutant HFE protein results in cellular overload, one would expect the microvasculature of the brain to have downregulated iron uptake machinery, such as transferrin receptor (TfR), when compared to wild type. This downregulation would be necessary to explain the similar rates of brain iron uptake. Knowing the cellular localization of TfR or the HFE protein could help to understand the Tf cycle through the endothelial cell and also determine if iron can move out of the brain via the BBB or if iron movement at the BBB is unidirectional.

5.3. Are There Conditions Where Iron Supplementation Works?

That the brains of people with RLS have lower than normal iron levels has been well-characterized (Allen et al. 2001; Allen and Earley 2001; Connor et al. 2011; Earley et al. 2006). Moreover, RLS occurs in 1/3 of people with anemia (Allen et al. 2013). Therefore, RLS is an opportunity to study as a model of adult brain iron deficiency. Of note, dopamine agonists have been found to be effective treatments for RLS symptoms, which suggests that the dopamine system is functionally impaired (Akpinar 1982; Allen and Earley 1996; Benes et al. 1999; Thorpy 2005; Trenkwalder et al. 1995; Walters et al. 2004) which would be consistent with brain iron deficiency as we noted earlier.

In addition to dopamine agonist treatment, intravenous (IV) iron therapy has been used successfully to treat RLS (Allen et al. 2013; Cho et al. 2013; Oski et al. 1983; Wang et al. 2009). How does successful treatment of RLS symptoms reconcile with our finding that CSF from RLS patients had no significant impact on iron movement across the BBB; unlike the CSF from iron
deficient monkeys (Simpson et al. 2015)? When iron deficient adult rats are given IV iron, iron levels were replenished in those regions of the brain that were iron deficient. The iron deficiency only occurred in specific regions of the brain that are associated with the nigrostriatal pathway, including the ventral midbrain and the nucleus accumbens (Unger et al. 2013). Those regions of the brain such as the prefrontal cortex and caudate putamen did not become iron deficient in this model and did not accumulate additional iron (Unger et al. 2013). In an additional study evaluating dietary iron repletion, iron deficiency was only observed in the cortex and the hippocampus (Erikson et al. 1997). In this study, the subsequent dietary repletion only increased iron in the depleted cortex and hippocampus and did not alter other regions of the brain such as the pons and thalamus (Erikson et al. 1997). Of note, these studies used different models. While the IV iron study evaluated deficiency repletion in female mice, the oral iron study studied male rats, which may highlight the importance of sex in brain iron uptake (explored below) (Erikson et al. 1997; Unger et al. 2013). These data demonstrate a regional selectivity to vulnerability to loss of iron and responsivity to replenishment which would be consistent with our findings of local regulation via apo-Tf, which would be more prevalent in an iron-deficient milieu. Therefore, in the case of RLS, responsiveness to IV iron may be reflective of increased levels of apo-Tf in the nigrostriatal brain environment rather than uptake to meet the global brain iron deficiency. A local response would explain the improvement seen in RLS with IV iron therapy – the iron could be taken up in only the nigrostriatal pathway to address components of the disease, such as dopamine system dysregulation. But, the IV iron treatment does not appear to improve the hypomyelination associated with RLS and is more reflective of a global brain iron deficiency.

A final consideration for responsiveness to IV iron therapy is sex. RLS has increased prevalence in the female population, including during pregnancy (Berger et al. 2004; Bjorvatn et al. 2005; Lavigne and Montplaisir 1994; Rothdach et al. 2000). As was already mentioned, we presented
data showing that female mice take up more iron than males; even though their total brain iron levels are similar. Thus, sex may be a final, but crucial, consideration before administering IV iron treatment for RLS patients. Our data suggest that females may be more responsive than males to IV iron treatment. Alternatively, females may take up more iron to address the differences in metabolic needs between the sexes. For example, females have higher rates of dopamine synthesis (Andersen et al. 1997; Bazzett and Becker 1994; Becker 1990; McDermott et al. 1994; Munro et al. 2006) and increased myelination (Bayless and Daniel 2015; Yang et al. 2008). Therefore, females may need additional dosing to meet the higher rates of iron utilization. These findings support the need to investigate the role of sex in iron supplementation responsiveness. In our present study, we only investigated the impact of CSF from female patients on movement of iron across an in vitro model of the BBB. It would be beneficial to expand this study to include both control and RLS male patients. This study would address two questions: 1) Does the male brain signal differently from the female brain to induce brain iron uptake? 2) Does the male brain alter its signaling profile in response to the brain iron deficiency observed in RLS in an attempt to correct the deficiency? Additionally, clinical data from RLS patients may provide valuable answers to this question in a human model. Key pieces of information would include responsiveness to IV iron treatment of males and females based on resolution of symptoms as well as magnetic resonance imaging to determine if the regional brain iron alterations observed in RLS are corrected by the IV treatment.

5.4. Is the Nigrostriatal System the Only Area Where Iron Status is Alterable?

The nigrostriatal pathway has been perhaps the most intensely studied area in the brain. It is the site of iron accumulation in at least two neurodegenerative diseases; including neurodegeneration with brain iron accumulation (NBIA) and Parkinson’s Disease (PD) (Berg 2006; Dexter et al.
In mouse genetic studies it was revealed that iron accumulation in this region has a genetic component that can result in as high as a 3-fold difference in iron content (Jones et al. 2003).

Although regional regulation of iron, particularly in the nigrostriatal pathway, is clearly possible and consistent with our data that apo-Tf is a signaling mechanism, a consistent finding in studies involving developmental iron deficiency is that the ID brain is hypomyelinated. The impact of hypomyelination on cognitive and motor performance is measurable and is not responsive to iron supplementation (Algarin et al. 2013; Corapci et al. 2006; Dallman 1986; Roncagliolo et al. 1998). Why are white matter tracts persistently impacted by lack of iron availability but the nigrostriatal system is more responsive? The cells that stain for iron in the brain, regardless of brain region, are primarily oligodendrocytes. This includes the iron-rich substantia nigra where the bulk of stainable iron is in the pars reticulata (Connor and Menzies 1996; Hill et al. 1985; LeVine and Macklin 1990). We have demonstrated that iron transport can be modulated by apo-Tf and the availability of apo-Tf would be in grey matter rich areas of the brain because it is the neurons that use Tf for iron uptake and would be recycling apo-Tf after iron uptake (Graeber et al. 1989; Swaiman and Machen 1984; Swaiman and Machen 1986). The oligodendrocytes of the white matter take up iron through ferritin rather than transferrin-based iron uptake (Todorich et al. 2009) and ferritin is likely internalized and degraded rather than recycled like Tf (Hulet et al. 1999a; Hulet et al. 2002; Hulet et al. 1999b). Thus, regional regulation of iron uptake may be driven by the metabolic activity of neurons and through signaling by recycled apo-Tf from the neurons after delivering iron. If transferrin-mediated uptake can occur on a local level, grey matter cells, such as dopaminergic neurons, can obtain iron and rescue their deficient phenotype. Meanwhile, if oligodendrocytes of the white matter obtain iron through ferritin, the presence of
apo-Tf may not allow for brain iron recovery. Therefore, myelin changes may be reflective of a global brain iron problem that is resistant to supplementation strategies.

In studies of iron deficiency, the nigrostriatal pathway appears to lose iron more readily than other brain regions. Therefore, this brain region may be more responsive to systemically delivered chelation therapy. Indeed, Deferiprone (DFP), a BBB permeable iron chelator, was shown to reduce only nigrostriatal-associated iron measured by magnetic resonance imaging in NBIA after 6 months of treatment (Abbruzzese et al. 2011; Forni et al. 2008; Zorzi et al. 2011). Additionally, the decreased iron measures were correlated with attenuated symptoms such as gait abnormalities and orofacial dystonia (Forni et al. 2008; Kwiatkowski et al. 2012). A clinical trial testing DFP therapy in PD also demonstrated decreased iron in the substantia nigra accompanied by improved motor function (Devos et al. 2014; Grolez et al. 2015). These studies all point to regional regulation of iron in the substantia nigra.

Recently, a study by Baksi, et al. implicated alpha-synuclein (α-syn) in the uptake of TBI into the epithelial cells of the blood-retinal barrier (BRB) (Baksi et al. 2016). In this study, α-syn was expressed widely in the neuroretina (Baksi et al. 2016). Furthermore, knockout of α-syn caused significant reductions in ferritin in the neuroretina as well as organs associated predominantly with uptake of TBI such as the spleen and, importantly, the brain (Baksi et al. 2016). Organs associated with non-TBI uptake did not exhibit the same reductions in ferritin. As previously noted, the substantia nigra develops elevated levels of iron in PD. Interestingly, overexpression of α-syn corresponds with the regional elevations of iron observed in PD (Shulman et al. 2011; Stefanis 2012). This new study implicating α-syn in TBI uptake at the BRB further supports the potential for local signaling to alter iron transport at physiological barriers and similar studies on
the BBB may be informative in understanding how and why there is increased iron in the substantia nigra in PD brains (Berg 2006; Dexter et al. 1987; Dexter et al. 1989; Sofic et al. 1988).

5.5. Diseases with Too Much Iron – Is There Anything That Can Be Done?

Brain iron loading has been well-documented with both aging and many brain diseases. Our laboratory has used the H67D/H67D knock-in mouse to study the role of a genetic mutation, which predisposes cells to iron overload, in brain disease pathology. Characterization of total brain iron in the H67D/H67D mice has documented an evolving brain iron status – at 3 months of age, the mice have ~40-60% higher iron than wild type; at 6 months of age, the mice have ~21% higher iron than wild type; at 12 months of age, there are no significant differences from wild type; at 18 months of age, the mice have ~40% higher iron than wild type (Ali-Rahmani et al. 2014; Nandar et al. 2013). In Chapter 3 we determined there is no significant difference in the rate of iron uptake into the brain of H67D/H67D mice at 3 months of age.

The similar rates of iron uptake despite documented brain iron elevation in 3 month old H67D/H67D mice raises a number of questions that can alter public health perceptions about iron supplementation and iron deficiency. First, what are the consequences of more iron in the brain? Are there greater amounts of myelin? More dopamine? Greater metabolic activity? Although we will address these questions in future studies, the similar levels of iron uptake suggest there is no significant increase in iron-dependent functions. Recently, a study showed decreased dopamine transporter and dopamine D1 receptor in H67D/H67D mice at 8 weeks of age (Chang et al. 2014). Surprisingly, these results are reflective of the changes observed in iron deficiency and further underscores the unique responses in the nigrostriatal pathway that should be further
explored. The data supports the notion of a developmental brain iron set point, in the case of this mutant mouse a higher than normal set point. In a setting where the set point is elevated rather than decreased, it is interesting to note that iron uptake was not decreased relative to control. These data along with the decreased efficacy of supplementation for correcting brain iron deficiency strongly argue for the set point concept and fixed signals from the brain that maintain the brain iron concentration thereafter.

Our age-based characterization of the H67D/H67D mice indicates that iron loading observed at 3 months levels off by 12 months of age as the normal amount of age-related iron accumulation “catches up” to the mutant. However, after 12 months of age, excess iron begins to accumulate again and is elevated compared to wild type by 18 months of age (Ali-Rahmani et al. 2014; Nandar et al. 2013). The observed elevation at 3 months of age without increased rate of iron uptake indicates one of two possibilities: 1) Iron had greater access to the brain at an early age and is consistent with the high iron needs of the developing brain. 2) A difference in iron export from the brain could be occurring in the HFE mutant mice. The wild type HFE protein has been shown to prevent cellular iron efflux from macrophages (Drakesmith et al. 2002) but we have shown that ferritin staining is elevated in the microglia in the mutant mouse brain (Nandar et al. 2013). The data suggest that iron efflux is altered in the presence of the HFE mutation although we cannot rule out that iron uptake increases after 12 months of age. Nonetheless, it appears that our HFE mouse strain could be a significant new model for study of iron efflux from the brain; about which very little is known.
That there is no longer a difference between wild type and H67D/H67D brain iron by 12 months suggests that the brain adapts to the elevated iron. But, by 18 months, the adaptive mechanism has been lost. The changes in the brain with aging in the H67D mutant mouse are similar to that seen in MRI of humans with the similar mutation (Meadowcroft et al. 2015). Aging is a key contributor to brain iron status. In fact, numerous studies have reported gradual increases in the normal adult brain with age (Bartzokis et al. 2010; Connor et al. 1990; Connor et al. 1995; Hallgren and Sourander 1958; Pirpamer et al. 2016; Zecca et al. 2004). Moreover, as mentioned, there is increased iron in the brain in the humans with HFE mutations suggesting that these mice represent an exciting translational model for interrogating brain iron uptake mechanisms and signaling with age and the role of HFE mutations in age-related neurodegenerative disease.

The studies with H67D mice introduce, as we previously mentioned, the need to better understand brain iron efflux. That the brain does not attempt to alter brain iron uptake in response to increased brain iron levels nor does it continue to load iron suggests that signaling to the BBB normalizes similarly between the two genotypes. Therefore, the “catching up” observation made from 3 months to 12 months in our model may implicated increased brain iron efflux in the H67D mouse. If the brain is not correcting its brain iron imbalance at the level of the BBB, is it correcting itself through efflux? Studies of the glymphatic system may provide answers to this question. A study by Kress, et al. demonstrated that clearance from the brain decreases with age (Kress et al. 2014). Therefore, in the H67D brain where brain iron efflux may be the only source of brain iron regulation, the decreases with age may be further affected. To this end, studying the efflux of iron from the brain is crucial. As with other glymphatic system studies, the $^{59}$Fe could be infused into the brain parenchyma. After infusion, brain iron efflux could be detected by measuring the appearance of $^{59}$Fe into the bloodstream. Furthermore, it may be interesting to
utilize an iron isotope with a different spin state to utilize MRI to monitor brain iron uptake from an IV injection and then brain iron efflux after uptake.

### 5.6. How does sex affect brain iron uptake?

We have previously explored the sex differences in brain iron utilization. The question still remains, though, how the male and female brains would respond to similar insults. Because the female brain has higher iron utilization, would the female brain be more impacted by brain iron deficiency? The effect of sex on the extent of impact of iron deficiency on cognitive development has received little attention. In one study, no sex effect on cognitive inhibitory control was identified 10 years after iron deficiency, but this could be the result of low female sample size (male n=65, female n=4 for formerly iron deficient children) (Algarin et al. 2013). Our data suggest considering sex will add a novel and critical piece of public health information to the discussion of iron supplementation during development. This observation suggests that longitudinal studies evaluating cognitive impairment in adults that were iron deficient during development should be further striated by sex. Do females retain higher levels of cognitive and behavioral deficit than males after the early insult of iron deficiency? In addition to cognitive effects, the overall morphology of the brain may provide valuable insights into the sex effect on brain iron. If the female is more affected by iron deficiency, what changes are seen? In these studies, the size of the brain as well as the development of the brain (processes like myelination that require iron) should be evaluated based on sex. One would expect that the female would have smaller, less myelinated brains if the insult was greater. One study directly relevant to this latter idea reported that the male total brain volume, white matter volume, and grey matter volume was larger than the female brain (Groeschel et al. 2010). Interestingly, females also had a lower ratio of white matter-to-grey matter that was age dependent (Groeschel et al. 2010). Thus, iron
deficiency during development could cause the white matter-to-grey matter ratios to be even
greater between the sexes.

5.7. Conclusions

My thesis has challenged some established concepts and generated novel hypotheses that can expand the understanding of brain iron uptake. These are:

1) We have developed a mouse model in which brain iron is genetically increased but the rate of iron uptake is not. This model will provide insights into brain iron utilization in development and brain iron efflux with aging.

2) The mouse model, and existing data, suggests that iron accumulation during development must reach a critical level and accumulation beyond that has little impact on brain size or function. The data also indicate that there is little utilization of the additional iron because rates of iron uptake are not increased to maintain the elevated iron. These data may critically impact public health policy regarding iron supplementation during development. We need to determine if rates of uptake are increased in models of iron deficiency. We have shown they are not decreased with iron overload.

3) The mouse studies indicate that rates of brain iron uptake in female mice are much greater than male mice and suggest that females may be more vulnerable to cognitive effects associated with brain iron deficiency than males.

4) The lack of increased iron transport from RLS CSF in the BBB model suggests that brain iron deficiency in RLS brains could stem from improper signaling about the iron status of the brain.
5) Iron transport in the BBB correlated more strongly with hemoglobin levels than with serum ferritin and may offer insights into predicting responders versus non-responders to IV iron because ferritin levels are the current indicator for treatment.

6) The cell culture model revealed that apo-Tf increased the release of iron from the BBB model but not transport suggesting that the iron taken up from the blood is replenishing endothelial cell iron stores as suggested in our model.

7) Inhibition of DMT-1 decreases iron release by endothelial cells and delivery of the Tf-Fe complex. The latter suggests iron release in endosomes may help direct recycling vesicles to either the blood or brain side of the endothelial cells.

8) The in vivo and cell culture uptake studies suggest there is local regulation of iron release coordinated by local levels of apo-Tf in the extracellular space. This regional regulation may be driven by metabolic activity of TfR-rich neurons (Hill et al. 1985; Mash et al. 1990) in the nigrostriatal pathway that locally recycle apo-Tf. The local regulation versus the ability of the brain to take on additional iron after development will impact therapeutic strategies for treating brain iron deficiency.
5.8. References


Bazzett TJ, Becker JB (1994) Sex differences in the rapid and acute effects of estrogen on striatal D2 dopamine receptor binding Brain research 637:163-172 doi:http://dx.doi.org/10.1016/0006-8993(94)91229-7


Callahan LS, Thibert KA, Wobken JD, Georgieff MK (2013) Early-life iron deficiency anemia alters the development and long-term expression of parvalbumin and perineuronal nets in the rat hippocampus Developmental neuroscience 35:427-436 doi:10.1159/000354178


Christian P et al. (2011) Preschool iron-folic acid and zinc supplementation in children exposed to iron-folic acid in utero confers no added cognitive benefit in early school-age The Journal of nutrition 141:2042-2048 doi:10.3945/jn.111.146480


Crowe A, Morgan EH (1992) Iron and transferrin uptake by brain and cerebrospinal fluid in the rat Brain research 592:8-16
iron accumulation Movement disorders : official journal of the Movement Disorder Society 23:904-907 doi:10.1002/mds.22002


Hulet SW, Menzies S, Connor JR (2002) Ferritin binding in the developing mouse brain follows a pattern similar to myelination and is unaffected by the jimpy mutation Developmental neuroscience 24:208-213 doi:65704


Human Evolutionary Biology. Cambridge University Press,
Lavigne GJ, Montplaisir JY (1994) Restless legs syndrome and sleep bruxism: prevalence and association among Canadians Sleep 17:739-743
Munro CA et al. (2006) Sex Differences in Striatal Dopamine Release in Healthy Adults Biological Psychiatry 59:966-974 doi:http://dx.doi.org/10.1016/j.biopsych.2006.01.008
Nandar W, Neely EB, Unger E, Connor JR (2013) A mutation in the HFE gene is associated with altered brain iron profiles and increased oxidative stress in mice Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 1832:729-741 doi:http://dx.doi.org/10.1016/j.bbadis.2013.02.009


VITA

Kari Ann Duck

Education

2005-2009 Bachelor of Science, Biochemistry & Molecular Biology
Ursinus College, Collegeville, PA

2005-present Doctoral Candidate, Cellular and Developmental Biology Program
Pennsylvania State University College of Medicine, Hershey, PA

Honors and Awards

2016 Annual Blood-Brain Barrier Consortium Meeting Travel Scholarship Award

Professional Societies

2013-2014 Society for Neuroscience
2016-present International Brain Barriers Society

Publications


Oral Presentations


Poster Presentations


