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BRAIN IRON DEFICIENCY

IN HUMANS AND ANIMAL MODELS

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Neuroscience

by

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Abstract

The studies in this thesis were designed to investigate the consequences of iron deficiency in the central nervous system. Two different models of iron-deficient states were used for our investigation. Restless Legs Syndrome (RLS) was studied with a focus on iron misregulation. RLS is characterized by an irresistible desire to move the extremities, with the legs generally affected to a greater degree than the arms. We measured the levels of whole-molecule ferritin, H-ferritin, L-ferritin, and transferrin in the cerebrospinal fluid from patients with RLS, with the goal of attaining a profile of these proteins in the central nervous system. We also determined that the iron-signaling hormone hepcidin is present in the CSF, and that its levels are altered in RLS. To investigate the type of brain iron misregulation possibly occurring in RLS and its potential causes, we studied the short- and long-term consequences of early iron deficiency on rat brain. We utilized microarray analysis of the whole brain homogenate of the developmental iron-deficient rat model. The rats were born to iron-deficient mothers, and were analyzed at two different ages: at 21 days, while weaning and iron-deficient; and at six months, after a recovery period consisting of normal iron intake for five months. Over 300 genes were significantly changed in the 21-day animals. Several significant gene clusters of interest were identified, including: myelin-related, signal transduction, channel/pore class transporter and alpha-type channel activity, ion channel activity, DNA binding, transitional metal binding, and solute carrier family members. The impact of the misregulation of the genes in these clusters can cause decreased myelination and conductivity, as well as impaired cell-to-cell and intracellular signaling, in addition to the expected changes in iron-related genes. In the six month formerly iron-deficient animals, only twelve genes were identified as significantly changed, reflecting considerably fewer changes than the acute iron-deficient state, but changes that are long-term. Of the twelve genes found, all were down-regulated, and seven were either estimated sequence tags or transcribed sequences, leaving five genes whose function can be discussed. Of the five genes,

two are cytoplasmic, two are nuclear, and one is found in both the nucleus and cytoplasm. The down-regulation of genes from the six month animals represents weakened cytoskeletal stability and infrastructure of neurons, decreased nucleic acid translation, and decreased responsiveness to oxidative stress. Genes of interest from each age group were verified utilizing qualitative Real-Time PCR. Overall, the data suggest that iron deficiency irreversibly impacts a range of functions. Furthermore, the presence of gene changes in the six month animals after a five month period of iron recovery reinforce the importance of identifying the windows of opportunity for optimal intervention.

Our findings have had a significant impact on the current understanding of the relationship between the central nervous system and iron deficiency. We established a distinct CSF profile for RLS, provided novel information regarding the presence of hepcidin in the CNS, and found significant alterations in hepcidin levels in RLS. Additionally, we have identified numerous genes changed during and after iron deficiency in the developmental iron-deficient rat brain, providing the necessary first step in elucidating the biological basis of the behavioral alterations resulting from iron deficiency.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis (Lou Gherig's disease)
ApoE	apolipoprotein E
APP	amyloid precursor protein
ATP	adenosine triphosphate
ATPase	ATP hydrolysis activity
βA	beta-amyloid
BBB	blood-brain barrier
BCA	bicinchronininic assay
bp	base pairs
°C	Celsius
CNS	central nervous system
CO_2	carbon dioxide
Cu	copper
CSF	cerebrospinal fluid
DFO	desferoxamine
dH_2O	distilled (deionized) water
DMT	divalent metal transporter
DNA	deoxyribonucleic acid
FDR	false discovery rate
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron

FP	ferroportin
GABA	gamma aminobutyric acid
GCRC	General Clinical Research Center
H_2O_2	hydrogen peroxide
H-ferritin	heavy chain subunit of ferritin
HFE	protein product of the Hfe gene that is mutated in hereditary hemochromatosis
HH	hereditary hemochromatosis
HIV	human immunodeficiency virus
HRP	horse radish peroxidase
IL6	interleukin 6
IRE	iron responsive element
IRP	iron regulatory protein
K	potassium
kb	kilobase
kD	kilodalton
L-ferritin	light chain subunit of ferritin
LP	lumbar puncture
mRNA	messenger ribonucleic acid
MAP	microtubule associated protein
MM	mismatch
MRI	magnetic resonance imaging
MS	multiple sclerosis
MSA	multiple system atrophy

NPC	Niemann-Pick disease Type C
OD	optical density
$OH^?$	hydroxyl radical
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	Parkinson's disease
PLMS	periodic limb movements of sleep
PM	perfect match
RLS	Restless Legs Syndrome
RPM	rotations per minute
SAM	significance analysis of microarrays
SEM	standard error of the mean
SOD	super oxide dismutase
SSPE	subacute sclerosing panencephalitis
TBS	tris-buffered saline
TCA	tricarboxylic acid
Tf	transferrin
TfR	transferrin receptor
WT	wild-type

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Chapter 1

Brain Iron Regulation and Consequences of Brain Iron Imbalance

Cellular Processing of Iron

The capacity of iron to interconvert between ferric and ferrous states by readily accepting and donating electrons makes it bioavailable to cytochromes and hemoglobin, and as a cofactor for enzymes. This property of iron, however, illustrates the need for its tight regulation, as it can also catalyze free radical formation (Andrews 1999). Systemically, iron circulates bound to transferrin (Tf). Tf is largely produced in the liver and has a half-life of eight days (Finch and Huebers 1982). Within cells, iron is stored in ferritin (Ft), which is a large, 24 subunit protein consisting of light and heavy chains that can store up to 4500 atoms of iron (Harrison and Arosio 1996). The amount of iron uptake into a cell is largely dependent upon the amount of transferrin receptor (TfR) on the membrane, with each TfR molecule having the ability to bind two diferric Tf molecules (four Fe^{3+} atoms).

TfR and ferritin are regulated by cellular iron and cytoplasmic mRNA binding proteins known as iron regulatory proteins (IRPs) (Rouault 2001). The iron regulatory proteins (IRPs) interact with specific elements in mRNA transcripts referred to as iron responsive elements (IREs). Ferritin mRNA contains an IRE in the 5’ untranslated region (5' UTR), and transferrin receptor mRNA contains 5 IREs in the 3' untranslated region (3’UTR) (Leibold and Munro 1988; Mullner and Kuhn 1988). There exist two distinct IRPs, IRP1 and IRP2. The presence or absence of iron-sulfur clusters determines the functionality of IRP1 (Emerit, Beaumont et al. 2001). When the cluster is present — as occurs in conditions of sufficient iron supply — IRP1

acts as an aconitase, interconverting citrate and isocitrate. In states of iron depletion, the cluster is absent, and IRP1 takes on the role of a high-affinity binding protein for IREs. IRP2 does not contain an iron-sulfur cluster, and does not have aconitase activity. IRP2 is rapidly degraded under conditions of adequate iron supply (Kuhn 1998). Under low iron conditions, IRPs bind to the 5'UTR of Ft and prevent its translation. Concurrently, IRPs bind to the 3'UTR of TfR and act to stabilize its RNA for receptor translation. In the presence of sufficient iron, IRPs are released from both Ft and TfR, allowing for ribosomal attachment and subsequent translation of Ft as well as degradation of TfR mRNA (Rouault and Klausner 1997). This elegant system of IRP-IRE iron regulation allows for close control of cellular iron homeostasis by the iron molecule itself. The factors governing certain aspects of this system, such as iron-sulfur cluster synthesis, are not fully understood. It is possible that dysfunction of any aspect of the system by various mechanisms may occur or result in neurodegenerative disease. IRP/IRE interaction in the brain is disrupted in some individuals with Alzheimer's Disease (Connor, Menzies et al. 1992) and targeted deletion of the IRP-2 gene in mice may cause misregulation of iron metabolism and a neurodegenerative disease characterized by neuronal iron accumulation (LaVaute, Smith et al. 2001).

Hereditary Hemochromatosis and the HFE mutation

Iron overload brings adverse health consequences. The best known disorder of iron overload, hereditary hemochromatosis (HH), is likely the most prevalent Caucasian disease of monoallelic inheritance. HH is caused by one of several possible defects in the Hfe gene that are thought to result in a failure of the Hfe protein to effectively bind with the TfR protein at the plasma membrane. While the HH symptoms of fatigue, arthralgias, and liver disease are well

documented (Olynyk, Cullen et al. 1999; Levy, Montross et al. 2000), the effect of HH on the brain remains to be understood. Recent evidence suggests that the genetic Hfe mutation may also be linked to Alzheimer's Disease (AD) (Moalem, Percy et al. 2000; Sampietro, Caputo et al. 2001; Combarros, Garcia-Roman et al. 2003; Pulliam, Jennings et al. 2003; Robson, Lehmann et al. 2004) and Amyotrophic Lateral Sclerosis (ALS) (Wang, Lee et al. 2004), though there is still debate in the field (Candore, Balistreri et al. 2003; Berlin 2004; Yen, Simpson et al. 2004). The two most common mutations in HH worldwide are the C282Y mutation (1.9%) and the H63D mutation (8.1%) (Merryweather-Clarke, Pointon et al. 1997). The mutant Hfe genes in both ALS and AD may lead to an excess of redox-active iron and thus oxidative stress in neurons. The current proposed mechanism by which mutant Hfe generates oxidative stress suggests that the mutant Hfe protein is unable to associate effectively with the ß-2 microglobulin protein and TfR1, thereby failing to control iron and resulting in iron accumulation.

The Hfe protein may be part of the innate immunity. The mutation may also impact the inflammatory response and therefore may promote neurodegeneration by exacerbating cytokine mediated neurotoxicity (Connor 2004). The current thinking is that the Hfe mutation alone does not directly cause either disease, but rather may contribute to one or more aspects of the multifactorial acquisition of the diseases. In AD, for example, oxidative stress may be even further exacerbated in susceptible carriers of apolipoprotein E epsilon 4 (APOE4) (Robson, Lehmann et al. 2004). In ALS, not only are Hfe mutations present at a higher frequency in individuals with ALS than in those without this disease, cell models carrying an Hfe mutation show changes in the expression of tubulin, actin and Cu/Zn SOD in cells, consistent with the disruptions in axonal transport in ALS (Wang, Lee et al. 2004). While AD and ALS may share brain iron misregulation as a common feature, they have tremendously different symptomology

and disease progressions. These differences, as well as those in other iron-related neurodegenerative diseases such as Parkinson's disease (PD), Huntington's disease (HD), aceruloplasminemia, outline the diverse role of iron in the central nervous system, as well as the varied ramifications of dysfunction in iron transport and regulation.

Brain Iron by Region

The normal adult brain has a high concentration of iron (approximately 60 mg) in a distinct pattern of distribution – an observation that is not surprising, given the distinct cell types and functions of the brain. Most of the stainable iron and transferrin in the brain is found in oligodendrocytes. Transferrin receptor is found on astrocytes, blood vessels and large neurons in cortex, striatum, and hippocampus (Hill, Ruff et al. 1985; Mash, Pablo et al. 1990; Connor and Menzies 1995). Hallgren and Sourander (Hallgren and Sourander 1958) studied iron content in approximately 100 autopsy brains, establishing the standard reference for brain region iron quantification. They found the globus pallidus, the red nucleus, the substantia nigra and the putamen to contain the greatest amounts of iron, respectively, followed by the dentate nucleus, the caudate nucleus, and frontal white matter. They also noted that iron content is low in all brain regions at birth and increases to a fairly constant value by about age 40. Not surprisingly, regional iron content is disrupted in disease. In AD brains, iron levels in globus pallidus and frontal cortex are increased, as are transferrin levels in frontal cortex, compared to control (Loeffler, Connor et al. 1995). PD brains also contain abnormally high levels of iron compared to control in the globus pallidus. Further, the transferrin/iron ratio (a measure of the capacity to mobilize iron) in both AD and PD is decreased in globus pallidus and caudate (Loeffler, Connor et al. 1995). It is clear that there exists a very intricate system of iron distribution in the brain,

given the specific regional heterogeneity of iron in different areas of the brain and the number of different cell types in the brain with differing iron requirements (Burdo, Menzies et al. 2001).

More recently, high field magnetic resonance imaging (MRI) scanners (especially those operating at 3 T) have allowed for verification of the previous tissue studies as well as quantification of storage iron levels in living subjects (Bartzokis, Sultzer et al. 2000; Schneck 2004). For example, ferritin can be measured with specificity *in vivo* with MRI utilizing the field-dependent relaxation rate increase (FDRI) method. The method uses both high- and low-field MRI instruments and calculates the difference between the R2 measured with the two MRI instruments. In tissue, only ferritin iron is known to increase R2 in a field-dependent manner. Utilizing the FDRI method, Bartzokis et al found that ferritin levels are significantly increased on MRI in the basal ganglia of young-onset groups of PD and AD subjects compared to control groups, but not in older-onset patients, suggesting that elevated ferritin is a risk factor for age at onset of neurodegenerative diseases such as AD and PD (Bartzokis, Tishler et al. 2004). Iron-dependent MRI looks to be a very powerful biomarker for future diagnoses of iron-related neurological diseases, as it is capable of identifying the presence as well as the progression of these iron-related CNS disorders from a whole-brain perspective in live patients.

Brain Iron Transport

Much is known about the regulation of iron uptake and transport within the systemic circulation (Andrews 2000), but less is understood about those compartments within organs that are physically separate from the systemic circulation and therefore cannot acquire iron directly from serum transferrin. Among these compartments is the central nervous system (CNS). Unlike endothelial cells of the systemic organs, the cells of the brain blood vessels are joined by

tight junctions that present a physical barrier to the passage of proteins and molecules. Little is definitively known about the uptake and export of iron within the brain, or about its carrier state and method of distribution within the CNS, but it seems clear that the behavior of iron within the CNS is quite different from that within the systemic organs.

Many scientists regard the question of brain iron transport as one that has already been solved for several years, believing the mechanism of brain iron transport to mimic other regions of the body in utilizing the well-described transferrin (Tf)-transferrin receptor (Tf receptor) system. The role of transferrin in CNS iron regulation is not as straightforward as Tf in the systemic circulation. Iron and transferrin distribution in the brain are seemingly paradoxical, in that the areas of highest iron concentration – the globus pallidus, substantia nigra, red nucleus, dentate gyrus, thalamus, and putamen – have relatively low transferrin concentrations. The highest transferrin concentrations are found in the hippocampus and cortical region, and these areas demonstrate relatively low iron levels. Notably, the areas of highest iron concentration are associated with several neurodegenerative diseases, including Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS). Recent data from Zerpa et al (2000) may help in understanding this apparent paradox. They analyzed the regulation of transferrin expression in a human oligodendrocyte cell line and noted that while transferrin is synthesized in oligodendrocytes, it does not appear to be secreted by oligodendrocytes (de Arriba Zerpa, Saleh et al. 2000). Further, transferrin was localized in the cytosol and not in the secretory compartment, and this transferrin localization was correlated with the synthesis of an alternatively spliced transcript lacking the signal peptide sequence. Zerpa et al propose that such findings suggest that transferrin synthesized in the brain is

functionally different than transferrin elsewhere in the body, and that transferrin plays a role other than iron transport in oligodendrocytes and myelination.

Early work on the role TfR at the blood-brain barrier by Pardridge et al (Pardridge, Eisenberg et al. 1987) found that the TfR was not only present on isolated human brain capillary, but also provided evidence for TfR-mediated endocytosis into endothelial cytoplasm via acid wash studies. In this early work, Pardridge proposed that unlike most endocytotic transport systems, transferrin remained associated with iron. It is clear, however, that such a system is both insufficient and problematic when applied to the blood-brain barrier (Burdo, Menzies et al. 2001; Qian and Shen 2001). Also, Tf-independent brain iron transport has been demonstrated (Malecki, Buhl et al. 2000) and transport of iron within the brain may also be Tf-independent (Beard, Wiesinger et al. 2005). There is evidence outside the central nervous system for a Tf-independent iron transport system: mice and humans that lack Tf, for example, show iron overload in some parenchymal tissues despite their overall anemia (Goya, Miyazaki et al. 1972; Bernstein 1987).

Any model of transport must account for the whereabouts of Tf: does it cross the abluminal membrane and enter into the brain with iron, or does it remain within the endothelial cell? Studies of Tf brain transport have shown conflicting results. One in vivo rat study demonstrated that as much as 50% of I^{125}-labeled Tf was transported to the brain (Fishman, Rubin et al. 1987), indicating a transcytosis mechanism, while another study found that only 10% of the Tf reached the brain (Roberts, Fine et al. 1993), suggesting that a simple transcytosis mechanism is not sufficient for brain iron delivery. Such confusion led our laboratory to utilize fluorescein-labeled Tf along with radioactively labeled Fe^{59} in a bovine retinal endothelial cell culture system to investigate the mode of iron and Tf transport (Burdo, Antonetti et al. 2003).

Using Transwell culture inserts, we found that iron is transported into the basal chamber in both in its free form and bound to Tf. Non-Tf-bound transport of iron could be altered by iron-loading the cells or by treating them with ammonium chloride to prevent iron release with the endosome.

The observation that transferrin receptors in brain are located primarily within gray matter areas rather than in the iron rich white matter tracts further supports the role of transferrin-independent systems. Hulet et al (Hulet, Powers et al. 1999) demonstrated the presence of ferritin binding sites in normal human brain in white matter tracts in a distribution opposite of that seen for transferrin receptor, providing evidence of ferritin binding in human brain. They also found that in Multiple Sclerotic patients the normal pattern of transferrin and ferritin binding distributions is disrupted, with ferritin binding absent in the lesion and the periplaque region in the white matter.

The traditional thinking for brain iron uptake whereby TfR bound to Tf molecules travels across the lumen poses several potential difficulties for interpreting transport of iron across the blood-brain barrier. First, it is unclear how the Tf-TfR complex is processed between the luminal and abluminal membranes. If the TfR-Tf-iron complex is transcytosed to the abluminal membrane, Tf should not be able to dissociate from TfR until the Tf first releases its iron. It is implausible that the Tf would release its iron without a significant alteration of the dissociation constant (estimated to be approximately 10^{-22} M), which requires a reduction in pH (Aisen and Listowsky 1980). Acidification could be accomplished in a model that favors endocytosis rather than transcytosis, as the pH-labile endosomes are the established intracellular site for iron release. A plausible model of endosomal brain iron transport involves Tf delivery of iron by binding to cell-surface Tf receptors, which are then internalized into endosomes. The iron is

then released from the TfR at the lower endosomal pH and is transported out of the endosome by divalent metal transporter-1 (DMT1, also known as DCT1, Nramp2).

DMT1 in the brain

DMT1 was first recognized in iron transport for its role in transferrin-independent intestinal iron absorption. DMT1 is located on the apical villi of enterocytes in the intestine, and its role here is to take up ferrous iron in the intestinal lumen, specifically the duodenum (Fleming, Romano et al. 1998; Canonne-Hergaux, Gruenheid et al. 1999). DMT1 has been demonstrated in brain endothelial cells (Burdo, Menzies et al. 2001), lending support to its role in blood-brain barrier transport. Interestingly, DMT1 exists in at least four different isoforms. Two forms differ at 3' end of the mRNA (IRE+ and IRE-), and the other two forms differ at the transcription start site on the 5' N-terminus. The different forms of DMT1 are distributed in different compartments of the cell, with the -IRE form of DMT1 found in the nucleus and cytoplasm of neuronal and neuronal-like cells and the +IRE species found in the cytoplasmic compartment of these cells, suggesting that the two proteins may have different functions in vivo (Roth, Horbinski et al. 2000). One study comparing differences in expression and localization of the isoforms in rat astrocytes showed that neither the IRE+ or IRE- isoforms co-localized with the TfR (Lis, Barone et al. 2004), possibly suggesting distinct, specialized functions for the DMT1 isoforms.

A valuable animal model for the study of DMT1 is the Belgrade rat. This animal contains a defective DMT1 gene, resulting in impaired intestinal iron uptake and systemic iron deficiency, as well as lower levels of iron in the brain (Fleming, Romano et al. 1998; Burdo, Menzies et al. 2001; Zywicke, van Gelderen et al. 2002). Recent work by Moos and Morgan

(Moos and Morgan 2004) investigated the role of DMT1 in brain iron transport utilizing the Belgrade rat model. They found that the DMT1 antibody they used detected the same levels of DMT1 in neurons and choroids plexus of both Belgrade (b/b) and control animals, but they failed to detect DMTl in brain capillary endothelial cells. From these data, they suggest "that the low cerebral iron uptake in b/b rats derives from a reduced neuronal uptake rather than an impaired iron transport through the blood-brain barrier" (Moos and Morgan 2004). They argue in favor of a transcytosis theory of iron transport that does not require the presence of DMT1 in brain endothelial cells, suggesting instead that there exist microenvironments (created by such mediators as citrate and ATP) in brain extracellular fluid that allow for iron release from Tf. DMT1, they propose, only plays a role at the neuronal level, not in endothelial cell iron transport. Burdo et al have shown, however, that both Tf associated and non-Tf associated iron is released from a cell culture model of the blood brain barrier (Burdo, Antonetti et al. 2003). The release of non-Tf associated iron strongly supports the presence of DMT1 in the endothelial cell. Furthermore, a purely transcytotic mechanism for transport of iron across the blood brain barrier ignores the iron requirements of the endothelial cells themselves, which have a high concentration of mitochondria that are required to produce the high levels of ATP for the transport activity of the mitochondria.

The inherited neurodegenerative disease Friedrich ataxia (FA) raises additional questions regarding iron transport and the role(s) of DMT1. FA is a condition characterized by progressive ataxia, dysarthria, sensory loss, and cardiomyopathy (Ponka 2002). Frataxin, the defective mitochondrial protein in FA, plays a role in mitochondrial iron metabolism, such that decreased frataxin levels result in iron loading and oxidative damage of the mitochondria. Becker et al (Becker, Greer et al. 2002) hypothesize that frataxin down-regulation may result in

mitochondrial iron accumulation by regulating (directly or via multiple proteins) metal transporter DMT1. Support for this idea is found in yeast, where the gene *CCC1* reportedly inhibits mitochondrial iron uptake. *CCC1* expression in cells increases expression of the yeast iron transport system, suggesting that intracellular iron is not sensed by the "iron-dependent transcription factor." The mechanism by which *CCC1* expression affects cytosolic iron is unknown, but the data suggest that excessive mitochondrial iron only occurs when cytosolic free iron levels are increased (Chen and Kaplan 2000). Becker et al (Becker, Greer et al. 2002) propose that frataxin could regulate a *CCC1*-type molecule in mammals.

Hepcidin

An unexpected recent candidate for involvement in a Tf-independent system is the protein hepcidin. Hepcidin was discovered as an antimicrobial hormone produced by the liver in response to inflammation, and it has since been found to limit iron availability to the body at the level of the gut (Pietrangelo 2004). Gene deletion of hepcidin results in iron loading of the liver (Nicolas 2001), while humans that express high levels of hepcidin as a result of liver adenomas suffer from a chronic anemia (Weinstein, Roy et al. 2002). Hepcidin over-expression results in a hypoferraemic, microcytic anemia that is refractive to iron (Dallalio, Fleury et al. 2003). In HH, hepcidin levels are low in iron-overloaded patients (Nemeth, Valore et al. 2003), an observation that led Pietrangelo and Trautwein (Pietrangelo 2004) to suggest that these inadequate circulating levels of hepcidin result in uncontrolled release of iron from the intestine and macrophages, producing the characteristic iron overload and damage associated with HH. Further, in the mouse model of HH, over-expression of hepcidin can correct the defect (Nicolas, Viatte et al. 2003). Identification of the targets and receptors for hepcidin is necessary to

understand fully its functions. Potential manipulation of its agonist and antagonist activity could be beneficial in treating iron-related disease - minimally anemia of chronic disease and HH. Presently, hepcidin has only provided evidence for the regulation iron absorption at the level of the gut, but future study of this protein is certainly warranted in the brain. In Chapter Three, I provide the first evidence for the presence of hepcidin in human CSF, and its potential role in brain iron signaling.

Hephaestin

Hephaestin, a membrane-bound ferroxidase, is another recently discovered player in systemic iron transport that warrants specific CNS investigation. The complete mechanism of action for hephaestin remains unclear, but its importance in iron homeostasis is illustrated by the sex-linked anemia (*sla*) mouse (Vulpe, Kuo et al. 1999). The sla mice have a mutant form of hephaestin and a defect in basolateral intestinal iron transport with resultant iron deficiency and anemia. Interestingly, when crossed with the mouse model of hemochromatosis (*hfe* mice), the *hfe/sla* mice accumulated less iron in the liver, demonstrating an inability of these mice to acquire as much iron as *hfe* mice (Levy, Montross et al. 2000).

At the amino acid level, hephaestin is 50% identical to ceruloplasmin, another ferroxidase that is found in plasma (Chen, Su et al. 2003). Mutations in the ceruloplasmin gene cause an autosomal recessive disease known as aceruloplasminemia. This disease is characterized by iron accumulation (including increased iron in the cerebrospinal fluid), and clinically is often manifest by diabetes and degeneration of the retina and basal ganglia (Qian and Shen 2001).

Ceruloplasmin

Ceruloplasmin (Cp) is serum glycoprotein belonging to a family of blue copper oxidases. The primary role of Cp appears to be as a ferroxidase, aiding in iron efflux from cells before binding to apotransferrin, but Cp also functions as an amine oxidase, an antioxidant, and a copper transporter (Qian and Wang 1998). Ceruloplasmin is mainly synthesized in hepatocytes, with a smaller amount being produced in the central nervous system most likely by astrocytes (Klomp, Farhangrazi et al. 1996). As mentioned above, mutations in the ceruloplasmin gene cause aceruloplasminemia. The cerebrospinal fluid of patients with aceruloplasminemia contains not only increased iron levels, but also increased peroxidation products and superoxide dismutase activity. Harris et al (1999) demonstrated that Cp knockout mice demonstrate significant impairment of iron export from reticuloendothelial cells and hepatocytes (Harris, Durley et al. 1999). Qian et al have also found early evidence for the role of CP in iron influx as well (Qian and Shen 2001). In the BT325 glioblastoma cell line, Cp was shown to promote iron uptake rather than release in both iron-sufficient and iron-deficient cells (Qian and Shen 2001).

Lactotransferrin

Lactotransferrin (Lf) is found normally in breast milk and saliva, where it serves as a potent antibacterial agent by binding free Fe^{3+} and denying it to invading bacteria. Lf is also found in the lesions of some neurodegenerative disorders, most dramatically in Guamian ALS-parkinsonism-dementia complex (Leveugle, Spik et al. 1994). This complex refers to the specific geographic isolate of motor neuron disease localized to Guam. Betz cells are immunoreactive for lactotransferrin in the affected areas of the ALS brain (Leveugle, Spik et al. 1994). The overproduction of lactotransferrin and its receptor has also been associated with degeneration of dopaminergic neurons in the substantia nigra in PD (Faucheux, Nillesse et al.

1995). Lactotransferrin receptor (LfR) immunoreactivity in the mesencephalon of PD brains was most pronounced in those areas with severe loss of dopaminergic neurons, and immunoreactivity intensity in the substantia nigra was greatest in those brains with greatest dopaminergic loss. Expression of lactotransferrin-positive neurons was decreased in PD, but of those neurons surviving, immunolabeling demonstrated higher Lf levels compared to control cases. Similarly, increased Lf expression has been found in AD, but LfR expression has not been studied, and again suggests the importance of further investigation focusing on Lf and LfR functions, mechanisms, and transport in both normal and diseased brains. No evidence to date has convincingly suggested a purpose for Lf in the brain, nor a mechanism by which Lf functions in the brain or gains access to the brain.

Melanotransferrin

Melanotransferrin (MTf) is also associated with AD via over-expression and may contribute to the excess iron accumulation seen in this disease (Jefferies, Food et al. 1996). MTf is an iron binding protein that was first identified on melanoma cells. The protein is encoded on chromosome three in humans, and while it is structurally similar to Tf and Lf, its function has not been well determined. MTf has been shown to be expressed on reactive microglial cells in AD patients and on the associated amyloid plaques in postmortem tissue. MTf was not expressed on those microglia not associated with plaques, and was not found in tissue from PD, HD, or ALS brains, indicating that MTf is a marker for AD (Jefferies, Food et al. 1996). Additionally, a correlation has been shown between increasing serum MTf concentration and disease progression, suggesting the role of MTf as a provider of the excess iron found in senile plaques (Kennard, Feldman et al. 1996). The distribution and expression of MTf and its

physiological role warrant further investigation. Further, the function of MTf must be definitively differentiated from Tf and Lf.

Iron-related Neurodegeneration

Many neurodegenerative disorders share several general pathogenic processes, including oxidative stress and free-radical activity, accumulation of intracellular aggregates, and mitochondrial dysfunction. An imbalance of iron concentration is perhaps a common mediator of these effects, thus making an understanding of its regulation essential to attaining a complete picture of neurodegeneration. The role of additional iron transport proteins in the CNS is one of growing importance, as it appears that such proteins as ceruloplasmin (Cp), lactotransferrin (Lf), melanotransferrin (MTf), and divalent metal transporter 1 (DMT1) exert a substantial influence on iron trafficking. The uneven distribution of these proteins among brain regions implies that iron transport is specific and regional within the brain, and further, increased or decreased production of some of these proteins is not surprisingly implicated in some neurodegenerative diseases. It has long been observed that iron accumulations are often present in the brains of sufferers of neurodegenerative disease, yet only recently have the mechanisms of these accumulations begun to be understood, and with this knowledge the realization of the primary importance of errors in iron metabolism and transport.

Many diseases of iron accumulation do not directly affect iron storage and transport molecules, but are the result of mutations in molecules that interact closely with iron and can affect its homeostasis. Hallervorden-Spatz Syndrome (HSS) is a rare neurodegenerative disease of childhood in which extra pyramidal dysfunction is characterized by rigidity, dystonia, and choreathetosis. The globus pallidus and substantia nigra of these patients have substantial iron

accumulation. The underlying mutation in a small number of patients with this disease appears to affect the PANK2 pantothenate kinase, an enzyme essential in coenzyme A synthesis and catalysis of the phosphorylation of vitamin B5 (pantothenate) in a cysteine-consuming reaction (Zhou, Westaway et al. 2001). PANK2 is specifically expressed in the brain, and its absence in these patients likely results in the observed cysteine accumulation in degenerating areas of the brain. A possible mechanism for iron-mediated damage implicates initial cysteine accumulation in the brain and resultant iron chelation by cysteine. It is notable that the causative mutation is a loss-of-function mutation and thus possible therapeutic strategies for this subset of HSS sufferers may target delivery of phosphopantothenate or coenzyme A.

Beyond those diseases for which a specific means of iron mishandling has been described, there exist several other potential mechanisms for production of iron-induced pathology. The condition known as neuroferritinopathy provides an example of a defect not in iron transport, but rather in iron storage (Curtis, Fey et al. 2001). In this dominant condition, the insertion of an adenine within the gene that encodes for the ferritin light chain polypeptide results in the replacement of 22 C-terminal residues with 26 novel amino acids. As a result, iron accumulates in the basal ganglia. The serum ferritin level is generally decreased in these patients, but it is not clear if this is a result of decreased ferritin synthesis, impaired ferritin release from cells, or altered functioning of the mutant protein in serum.

The pigment neuromelanin is known to increase in the brain with normal aging, and it is particularly abundant in the substantia nigra. Initially named neuromelanin because of its similarity in appearance to cutaneous melanin, neuromelanin has recently been established to indeed be a true melanin by electron paramagnetic resonance and metal analysis studies (Enochs, Nilges et al. 1993; Zecca, Pietra et al. 1994). Neuromelanin can interact with several organic

molecules, toxic compounds, and heavy metals, but binds iron particularly strongly. Neuromelanin has been implicated in the development of PD, as an increased rate of degeneration of highly pigmented neurons has been observed in the substantia nigra. The concentration of iron in the substantia nigra in PD increases by 30-35%, and this accumulation seems to occur within neuromelanin granules, as the concentration of iron in these granules is higher in PD patients than normal subjects (Sofic, Paulus et al. 1991; Good, Olanow et al. 1992). In normal subjects, neuromelanin may play a protective role by sequestering redox active iron atoms and thus preventing oxidative stress and neuronal damage. When free iron increases such that neuromelanin is saturated, however, neuromelanin may become cytotoxic, enhancing the formation of free hydroxyl radicals by redox activation of the ions. Because hydrogen peroxide can degrade neuromelanin, additional iron release could occur, accelerating neuronal death (Zareba, Bober et al. 1995).

As more is discovered about each of the iron transport molecules and mechanisms of iron storage, it is likely that their role in neurodegenerative diseases will become better understood. Two animal models, the Belgrade rat in which a defect in the divalent metal transport protein is associated with decreased brain iron acquisition (Burdo, Menzies et al. 2001; Zywicke, van Gelderen et al. 2002), and a ferritin knockdown mouse that has normal iron concentrations but a decrease in ferritin concentration (Thompson, Menzies et al. 2003), have considerable promise in elucidating the contribution of iron mismanagement to neurodegenerative processes. Additionally, a third animal model in which transgenic mice expressing the ferritin heavy subunit (H ferritin) within dopaminergic SN neurons also appears to be a promising tool for the study of iron. Initial work with these mice demonstrated that the transgenic expression of the heavy

ferritin subunit prevented dopaminergic SN cell loss associated with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Kaur, Yantiri et al. 2003).

Iron and Inflammation

The effect of inflammation on iron metabolism is widely accepted, especially in the example of anemia of chronic disease, but the processes underlying the relationship are poorly understood. One proposed mechanism for the role of iron in system-wide inflammation suggests that the TfR located on monocytes facilitates iron entry into the cell in early inflammation, while elevation of H ferritin has a prolonged iron sequestration effect in macrophages (Scaccabarozzi, Arosio et al. 2000). Inflammation can cause alterations in the blood-brain barrier (BBB) that could potentially result in increased iron influx, though the mechanisms governing iron and inflammation in the CNS are poorly understood. Iron influx and deposition secondary to inflammation is believed to be mechanism of damage in Multiple Sclerosis (MS) (Minagar, Toledo et al. 2004). It has been observed that cerebrospinal fluid levels of ferritin are increased in MS patients (LeVine, Lynch et al. 1999). The definitive mechanism by which elevated ferritin and the inflammatory response may promote demyelination is not clear. The ferritin binding protein in the brain is potentially involved, as there is a loss of ferritin binding in the oligodendrocytes of periplaque regions in MS brains (Pinero 2000, Hulet, 1999 #).

Secondarily, cytokines secreted during inflammation may alter IRP function, further catalyzing iron accumulation (Pinero, Hu et al. 2000). When cytokines are given to human monocytes/macrophages, IRP activity is down-regulated — a finding thought to model the down-regulation of IRP activity in patients with inflammation (Recalcati, Pometta et al. 1998). Additionally, nitric oxide (NO) and peroxynitrite (ONOO-), often present from endogenous

synthases or exogenous donors, appear to activate IRP-1 by attacking its Fe-S cluster, while also inactivating IRP-2 (which has no clusters). Though divergent, these actions may explain the links between iron homeostasis and the reactive nitrogen species found in macrophages involved in inflammation (Cairo, Ronchi et al. 2002).

Inflammation also appears central to plaque formation in AD. Alzheimer's disease is characterized by the formation of plaques throughout the brain. The principle component of these plaques, beta-amyloid (Aß), is derived from amyloid precursor protein (APP). APP is a transmembrane glycoprotein expressed in several types of mammalian cells that, when cleaved according to its major pathway, does not produce Aß. Iron availability, which itself can be affected by inflammation, may affect processing of APP, as APP mRNA contains a putative IRE based on sequence homology (Bodovitz, Falduto et al. 1995). Further, neuritic plaques in AD hippocampus demonstrate strong immunoreactivity for ferritin, with the ferritin accumulation being associated largely with reactive microglia (Grundke-Iqbal, Fleming et al. 1990; Connor, Menzies et al. 1992). The increased expression of IRP-2 in association with neurofibrillary tangles and neuropil threads supports the role of altered iron metabolism in the pathogenesis of Alzheimer's disease (Smith, Wehr et al. 1998). In addition, IRP/IRE interaction is altered in some AD brains. In the latter case, IRP is more difficult to dissociate from the IRE in AD brain tissue. The result of this abnormally tight association would be increased Tf receptor expression, decreased ferritin expression and increased cellular iron accumulation. All of these are consistent observations in AD brain tissue (Pinero, Hu et al. 2000). Further related evidence for the role of iron in AD is provided by magnetic resonance imaging of AD brains which demonstrate increased iron levels in the basal ganglia (Bartzokis, Sultzer et al. 2000).

Alzheimer's plaques also contain oxidized and nitrated proteins of neuroinflammation, as well as a surrounding layer of activated microglia and astrocytes. Inflammatory response markers, including cytokines, acute phase reactants, and proteases, are also present in the AD brain. Additionally, epidemiologic studies have demonstrated that anti-inflammatory agents and corticosteroids are associated with slowing the progression or delaying the onset of AD (Markesbery and Carney 1999). Activated microglia cells are significantly increased in the AD brain, and are capable of releasing several interleukins, including IL-1, IL-6, and the widely implicated tumor necrosis factor alpha (TNF-a) (Carpenter, Carpenter et al. 1993). Furthermore, the high ferritin content, of microglia is potentially dangerous to themselves and neighboring neurons, as oxidants such as superoxide anion, H_2O_2, and nitric oxide are able to trigger the release of iron directly from ferritin *in vitro* (Mehlhase, Sandig et al. 2005). Another related mechanism of damage in AD focuses on the ability of IL-1 to up-regulate the expression of APP. Such an up-regulation could increase Aß and in turn cause H_2O_2 accumulation, thereby resulting in an IL-1 induced self-propagation of free radicals and neuron degeneration secondary to inflammation (Goldgaber, Harris et al. 1989).

As mentioned earlier in this chapter, several studies have suggested that carrying a mutation in the protein that causes hemochromatosis, an iron overload disease, may place individuals at greater risk for Alzheimer's Disease or at least influence the age of onset of AD (Moalem, Percy et al. 2000; Sampietro, Caputo et al. 2001; Combarros, Garcia-Roman et al. 2003; Pulliam, Jennings et al. 2003; Robson, Lehmann et al. 2004). Although the brain has historically been considered protected from iron accumulation in hemochromatosis, this theory has not been systematically studied (Sheldon 1935) and the Hfe protein that is associated with hemochromatosis is expressed on the brain microvasculature (Connor, Milward et al. 2001).

Thus, there is reason to suspect that the presence of the Hfe mutation, the most common genetic mutation in Caucasians, could influence iron accumulation in the brain (Connor, Milward et al. 2001).

Recently, it has been discovered that the iron hormone hepcidin appears to serve as an intermediary between inflammation, iron, and host defense. Hepcidin inhibits iron transport into plasma from macrophages, hepatocytes, and enterocytes, decreasing the amount of iron available for hemoglobin and erythrocytes. Hypoferremia results, and chronically, becomes the anemia of chronic disease (also called the anemia of inflammation). Transgenic mice that overproduce hepcidin-I suffer from lethal anemia, and human patients that suffer from tumors over-expressing hepcidin exhibit severe anemia (Nicolas 2002; Weinstein, Roy et al. 2002). The cytokine IL-6 appears to stimulate the hepcidin response in inflammation (Ganz 2005). Experiments have shown that IL-6 induces hepcidin synthesis in hepatocytes during inflammation; accordingly, if IL-6 action is blocked by antibodies in primary human hepatocytes, hepcidin induction is also blocked. Experiments in humans found that urinary hepcidin levels increased approximately 7-fold after IL-6 infusion, and this increase was accompanied by a 30% decrease in serum iron and transferrin saturation (Nemeth, Rivera et al. 2004).

Oxidative Stress and Neurodegeneration

Beyond misregulation of iron trafficking as a primary cause of neurodegeneration, consideration must also be given to defects in antioxidant defense mechanisms. The etiology of neurodegenerative diseases remains elusive, yet the body of evidence supporting the crucial role of oxidative damage — both as a primary and secondary cause of disease — is increasingly

convincing. As the knowledge of the various mechanisms of oxidative stress grows, so too does the list of diseases in which oxidative stress has implications.

Oxidative damage refers to the injury that can ensue as a result of excess free radical production, either endogenous or exogenous, or from a reduced capacity to neutralize free radicals. A free radical, by definition, can be any independent molecule containing an unpaired electron. This unpaired electron makes such molecules highly reactive with macromolecular structures, potentiating tissue injury and homeostatic disruption. The most important mechanism of radical production in vivo is likely the decomposition of superoxide and hydrogen peroxide as catalyzed by transition metals (Young and Woodside 2001). Normal cellular function involves the production of free radicals, yet these free radicals represent a precarious balance between health and disease. An example of free radical balance is the signaling molecule nitric oxide (NO). NO is synthesized from arginine and oxygen by the enzyme nitric oxide synthase (NOS), and is secreted by macrophages to defeat bacteria. Under septic conditions, however, excess NO production can result in vasodilatation and hypotension, due to NO's other role in smooth muscle relaxation (Landar and Darley-Usmar 2005). Any failure in the decomposition or scavenging of these radicals can result in a wide array of consequences, with neurodegenerative diseases being perhaps the most devastating of these.

Huntington Disease (HD) is a neurodegenerative disorder related to defective free radical detoxification. HD is caused by a trinucleotide (CAG) repeat expansion, and is characterized by degeneration of the striatum, as well as disturbances in motor and cognitive functions (1993). While the generation of a toxic N-terminus fragment of unknown function known as huntingtin is likely the primary mechanism of pathogenesis, striatal iron accumulation has been noted in

presymtomatic Huntington's sufferers, and it is thought that generation of free radicals via this mechanism plays a role in the neurodegeneration (Dexter, Carayon et al. 1991).

Further evidence supporting the crucial role of oxidative damage in neurodegeneration is demonstrated by both Alzheimer's and Parkinson's diseases. Alzheimer's brains have an increase in iron without an accompanying increase in ferritin in certain regions of the brain, including the superior temporal gyrus and the frontal cortex. Such an abnormal iron accumulation could put cells at an increased risk for oxidative stress; perhaps not coincidentally, these areas of the brain often demonstrate severe histopathology in AD. Similarly, in Parkinson's disease (PD), the primary site of neurodegeneration and the death of dopaminergic neurons in the substantia nigra pars compacta – a site that also often has increased iron in individuals with severe PD. As in AD, ferritin expression is also decreased in PD. Of surviving neurons in PD, some may contain Lewy bodies, which are intracytoplasmic inclusions of neurofilaments, ubiquitin, and alpha-synuclein. It has been shown that alpha-synuclein aggregation can be induced in vitro by iron-related oxidative stress, providing further evidence for the linkage between impaired iron metabolism and pathologic intracellular aggregates in PD (Hashimoto, Hsu et al. 1999 ; Paik, Shin et al. 1999).

Oxidative stress plays a clear role in familial cases of amyotrophic lateral sclerosis (ALS). ALS is a type of motor neuron disease characterized by progressive muscle weakness and wasting. Its prevalence is about five in 100,000, with approximately 10% of cases being familial autosomal dominant ALS. Mutations in the copper-zinc superoxide dismutase (SOD1) gene are responsible for some forms of familial ALS, with greater than one hundred mutations having been described in the SOD1 gene on chromosome 21 (Andersen, Sims et al. 2003). SOD1 normally converts superoxide anion to hydrogen peroxide, but can also form toxic

hydroxyl radicals. The SOD1 mutations support an excitotoxicity and free radical damage basis of disease in familial ALS. The presence of iron in spinal motor neurons may make them especially susceptible to ALS-type degeneration by facilitating the generation of free radicals (Kasarskis, Tandon et al. 1995). Familial ALS represents only a fraction of all ALS cases, however, and a causal relationship for sporadic ALS is less clear.

Mitochondrial Dysfunction

The intermediary role of mitochondria is a crucial link between iron metabolism and oxidative stress in the context of neurodegeneration (Jenner 1993). Mitochondria are the major intracellular source of free radicals, and thus any damage to mitochondrial DNA or to nuclear DNA coding for mitochondrial proteins often has neurodegenerative implications. The primary role of mitochondria is to provide ATP by aerobic metabolism, and thus these organelles are found in particularly high concentrations in tissues with high aerobic activity, including the skeletal and cardiac muscle and brain. In addition to ATP production, mitochondria play a key role in the regulation of apoptosis. Mitochondria have their own circular, double-stranded DNA, with a given mammalian mitochondria containing from two to ten molecules of mitochondrial DNA (mtDNA). Of this DNA, 13 proteins form components of the respiratory chain and oxidative phosphorylation system (OXPHOS), with the remaining 70 proteins in this system being supplied by nuclear genes. Neurodegenerative disorders involving mitochondria can accordingly be divided into those caused by OXPHOS abnormalities of either mtDNA or nuclear proteins (pure myopathies, complex I and II deficiencies), those resulting in OXPHOS abnormalities caused by nuclear mutations coding for non-mitochondrial proteins (HD), and those caused by nuclear genes encoding mitochondrial non-OXPHOS proteins (Wilson disease,

Friedreich's ataxia, hereditary spastic paraplegia) (Orth and Schapira 2001). Still other diseases have unassigned mitochondrial involvement, including PD, AD, and ALS, as discussed in the previous section.

Friedreich's ataxia (FA) is a dramatic example of the consequence of oxidative stress in the context of mitochondrial dysfunction. FA is an autosomal recessive disorder with a prevalence of one in 30,000 live births. It is characterized by progressive ataxia, neuropathy, and cardiomyopathy resulting from the loss of the mitochondrial protein frataxin with resultant mitochondrial iron overload. An expanded GAA trinucleotide repeat on chromosome 9 is responsible for the decreased frataxin levels (Campuzano, Montermini et al. 1996). Neurons and cardiac muscle are affected to the greatest degree, a finding that correlates with the strong dependence of these tissues on a continuous demand for iron uptake as well as a high level of mitochondrial energy production. Mouse and yeast models of FA demonstrate decreased aconitase activity, suggesting a role for frataxin in mitochondrial-cytosolic iron cycling. Antioxidant coenzyme Q and free radical scavenger idebenone represent potential cardiac therapeutics for FA patients (Rustin, von Kleist-Retzow et al. 1999). In a cellular model to determine the intracellular events associated with oxidative stress and iron, mitochondrial membrane potential and ATP production were both decreased when iron-loaded astrocytes were exposed to a pro-oxidant and were conserved in the presence of an iron chelators (Robb, Robb-Gaspers et al. 1999).

Potential Therapeutics

There remain a great number of unanswered questions regarding the mechanisms of neurodegenerative diseases, and accordingly there are few truly effective treatments for these

diseases, but many potential therapeutic targets. Little is known about the impact of dietary antioxidants on development and progression of neurodegenerative diseases, and past studies have been insufficient in design and methodology. The natural antioxidants, including vitamin E, the carotenoids, and the flavonoids, do not readily cross the BBB in adults, and thus other antioxidants, such as spin traps and low molecular mass oxygen scavengers, are being investigated (Halliwell 2001). Another possibility to be investigated is that some drugs already in therapeutic use for PD, such as selegiline and nitecapone, may owe some of their effects to antioxidant action.

Current treatment of Wilson's disease represents a prototype of neuroprotective therapy. Wilson's disease is a rare autosomal recessive disturbance of copper incorporation and resultant accumulation of copper in the brain, liver, kidney, and eyes. Decoppering therapy in such patients can slow or reverse the neurologic deterioration associated with copper accumulation. Such success suggests the importance of developing protective and preventive therapies for other neurodegenerative diseases associated with elemental iron accumulation, including AD, PD, and HD. One obstacle to overcome involves a primary difference between copper and iron excretion: there is an active, regulated mechanism of excretion for excess copper, but no such mechanism has been identified for iron (Andrews 2002).

The delivery of non-lipophilic compounds to the brain is limited by the blood-brain barrier. The transferrin receptor itself posses a potential route of therapy in neurodegenerative and other diseases, as antibodies that bind the TfR have been shown to selectively target BBB endothelium. Specifically, the OX26 antibody against rat TfR has shown potential for brain drug and gene delivery when conjugated to immunoliposomes (Shi and Pardridge 2000). Additionally, conjugates between OX26 and a variety of therapeutic agents - including

neuropeptides, polyamide nucleic acids, and nerve growth factor - have shown markedly increased delivery to the brain compared to IV administration alone (Kordower, Charles et al. 1994; Park, Starzyk et al. 1998).

To significantly improve clinical management of neurodegenerative diseases, genetic risk, susceptibility factors, and prodromal symptoms must be better characterized in order that preventive strategies can be targeted toward healthy subjects to postpone illness onset. Additionally, it is plausible that effective treatment of many neurodegenerative diseases would involve a bimodal approach, with both a reduction of brain iron levels as well as inhibition of free radical formation. Such a method could involve, for example, combination therapy with an antioxidant and an iron chelator. Both the antioxidants and the chelators must have high penetrability of the blood-brain barrier.

Brain Iron Deficiency

Iron is physiologically essential – a fact readily demonstrated by the syndromes associated with iron-deficient states, including iron deficiency anemia and cognitive deficits induced by developmental dietary iron deficiency. Given that insufficient body iron levels affect up to one billion people worldwide (Andrews 2000), and that even within the United States 3% of toddlers and up to 5% of adolescent females suffer from iron deficiency anemia (Looker, Orwoll et al. 1997), it is crucial to fully understand the effects of iron deficiency on the brain. Iron is especially crucial during development, with the effects of early dietary iron deficiency on the brain being largely irreversible. Given that iron is required for proper myelination of the spinal cord and white matter of cerebellar folds, and is also a cofactor for enzymes in neurotransmitter synthesis (Larkin 1990); (Ben-Shachar, Finberg et al. 1985; Erikson, Jones et al.

2001), it is not surprising that early iron deficiency can have long-term neurological effects. In the body of the thesis, evidence is presented to suggest that the adult onset sensorimotor disorder Restless Legs Syndrome (RLS) represents a type of iron insufficiency at the level of the brain, perhaps related to early iron deprivation (Connor, Wang et al. 2004; Wang, Wiesinger et al. 2004).

Studies dating back over three decades have found the signs of generalized neurological damage resulting from iron-deficiency. Dallman et al demonstrated that while hematocrit, liver non-heme iron, and liver ferritin values promptly returned to normal values after 28 day or 48 day iron-deficient rats were treated with iron for several days, non-heme iron remained depressed (19-29% below), as did ferritin in brain (33-42% below) (Dallman, Siimes et al. 1975). Weinberg et al also noted that rats exposed to iron deficiency for the first 28 days of life never recovered, exhibiting a persistent deficit (22%) in brain non-heme iron in adulthood coupled with long-term effects on behavior and physiological responsiveness, as measured by testing in an exploratory task and active and passive avoidance learning (Weinberg, Levine et al. 1979). Further behavioral effects, specifically in the dopaminergic system, were found in another study of rats that were iron-deprived for the first 28 days of life. The rats exhibited decreased motor activity and reversed circadian rhythms of thermoregulation and motor activity. Additionally, the hypermotility effect of d-amphetamine was reduced, and the hypothermic effect of d-amphetamine was significantly reduced, in correlation with the decrease in the blood and brain. Finally, when given apomorphine, stereotyped behavior was increased during the light period compared to controls. This study is indicative of the major role iron plays in the normal function of the dopaminergic system in the brain(Youdim, Yehuda et al. 1981). Biochemically, Mackler et al demonstrated decreased brain aldehyde oxidase (an enzyme in serotonin degradation), and

increased serotonin and total 5-hydroxyindole compounds in the brain tissue of iron-deficient animals, with recovery to near normal levels after a week or more of iron therapy (Mackler, Person et al. 1978). At the cellular level, studies of iron deficiency often focus on the oligodendrocyte, as it is the predominant iron-containing cell in the brain and is also the cell responsible for myelination (Hill 1988; Connor and Menzies 1996). In iron deficiency, oligodendrocytes appear immature (Erikson, Pinero et al. 1997), and disruption of oligodendrocyte maturation (as caused by some gene mutations) results in iron accumulation that is only 50% of normal levels (Connor and Menzies 1990).

The results of the animal studies have clear parallels in humans. A study of second grade children who were anemic as infants (n = 20) compared to children who were non-anemic in infancy (n = 55) revealed that the learning achievement score was significantly lower in the anemic group 9.3 S.D. 3.8, than in the non-anemic group 11.5 S.D. 2.9 (P = 0.009). Further, the positive task orientation was significantly lower in the anemic group. Data was controlled for maternal education and sex of child (Palti, Meijer et al. 1985). A more recent study by Lozoff et al found that children who had iron deficiency in infancy (n = 48) scored lower on tests of mental and motor functioning as teens (greater than ten years later) than those infants who were not iron-deficient as infants (n = 114). Additionally, more of the previously iron-deficient infants had repeated a grade, been referred for special services, and/or was the object of parental and teacher concern regarding anxiety/depression, social difficulties, and attention problems (Lozoff, Jimenez et al. 2000).

Iron-deficiency lowers brain iron and likely interferes with protein synthesis in the brain of animals. Given that the highest brain concentrations of iron are found in dopaminergic structures, and the crucial role of dopaminergic systems in attention and learning (Youdim, Ben-

Shachar et al. 1983), the behavioral changes from the reduction in dopaminergic activity in iron-deficient animals could explain the adverse effects on "cognition, behavioral patterns, learning and attention, event-related potentials (ERPs) and EEG changes reported in iron-deficient children" (Youdim, Ben-Shachar et al. 1983). There is a notable lack of research into the effects of such early iron deficiency in later, adult life. This is surprising, given the clearly dramatic changes in brain development observed through childhood. To investigate the long term effects of early iron-deficiency, we studied the developmental iron-deficient rat, and our findings are discussed extensively in Chapter Four. Briefly, the rats are the offspring of mothers who were iron-deficient through pregnancy and the weaning process. Post-weaning, the rat pups are on placed on an iron-normal diet. Using microarray analysis, we studied two age groups — six months of age (recovered iron-deficient) and 21 days of age (current iron-deficient) — to obtain a gene profile in the brain to identify the compensatory gene profile in young animals as they attempt to adapt to insufficient iron levels and to identify the long term consequences of developmental iron deficiency.

Restless Legs Syndrome

Restless Legs Syndrome (RLS) is an example of a disorder consistent with brain iron deficiency. RLS is characterized by an irresistible desire to move the extremities, with the legs generally affected to a greater degree than the arms (Earley, Allen et al. 2000). RLS sensations generally occur when the sufferer is at rest, and are relieved by voluntary movement. Suppression of these sensations, referred to as dysesthesias, only increases patient discomfort, often making activities such as plane travel or dining out extremely unpleasant. A circadian pattern characterizes RLS, with symptoms worsening in the evening (Walters, Aldrich et al.

1995). Indeed, a significant number of RLS sufferers are initially diagnosed based on interviews with the patient's bed partner. Symptom severity varies widely. The majority of RLS sufferers demonstrate periodic limb movements of sleep (PLMS) and consequently suffer from sleep deprivation. PLMS occur in 70%-90% of RLS patients, though at most only 30% of patients with PLMS will have RLS. Total daily sleep times are often reduced to 5 hours or less, with marked reduction of sleep efficiency (Sun, Chen et al. 1998). RLS sufferers are sleep deprived not only because they have difficulty in sleep maintenance, but also because they generally have a prolonged latency to sleep onset. This delayed sleep onset is not surprising, as trying to relax triggers symptoms.

Estimates of the prevalence of RLS range from 5 to 15% of the Caucasian population (Phillips, Young et al. 2000; Rothdach, Trenkwalder et al. 2000). RLS may be either primary, with no known relation to another disorder, or secondary to other medical conditions. Major causes of secondary and reversible RLS include pregnancy, iron deficiency and end stage renal disease, both before and after dialysis treatment (Callaghan 1966). Approximately 25 – 40% of the patients with any of these three conditions report RLS, which is about two to five times the expected occurrence for adults. Generally, RLS dramatically improves with the effective treatment or resolution of these conditions. Idiopathic RLS can begin at any age, but recent evidence suggests that the percentage of the population with RLS increases with age (Trenkwalder, Seidel et al. 1996; Bassetti, Mauerhofer et al. 2001). When symptoms begin before the age of 45, they are generally slowly progressive (Allen and Earley 2000), with the expression of the most severe symptoms occurring mainly in adults over the age of 50. The first-degree relatives of patients whose onset of symptoms occurs before age 45 are 5 times more likely to have RLS than the general population (Labuda 1997).

Monozygotic twin studies by Ondo et al have provided insight into the genetic and environmental factors involved in RLS phenotype expression. Ten of twelve twin pairs were concordant for RLS symptoms, yet severity, age of onset, and symptoms varied between the twins. Additionally, birth order, birth weight, and serum ferritin levels did not predict RLS severity or age at onset. Nonetheless, such high concordance supports the role of an autosomal dominant mode of inheritance for at least some cases of RLS (Ondo, Vuong et al. 2000). More recently, Trenkwalder et al have reported that alterations in a single, autosomal dominant gene may be responsible for most cases of early-onset RLS (EORLS). There was no such evidence for a similar mechanism in secondary RLS, indicating a clearly different mode of inheritance (Winkelmann, Muller-Myhsok et al. 2002).

The current clinical data clearly supports a role for the dopaminergic system in this disorder. Dopamine antagonists can aggravate RLS (Ekbom 1960) or induce a focal akathisia clinically similar to RLS (Braude, Barnes et al. 1983). Early clinical studies indicated that RLS was dopamine responsive (Akpinar 1982). Subsequently, low-dose levodopa treatment was found to be significantly more effective than the relatively high doses of an opiate that had previously been used to treat RLS (Montplaisir, Godbout et al. 1986; Allen, Kaplan et al. 1992; Kaplan, Allen et al. 1993). Levodopa was one of the first drugs to be proven efficacious in placebo-controlled trials. Treatment with levodopa is greatly limited, however, by augmentation of symptoms, including onset of RLS symptoms earlier in the evening compared to pretreatment onset, involvement of other body parts, and/or increased severity (Stiasny, Wetter et al. 2000). The dopamine agonist, pergolide, has since been found to provide effective treatment with less adverse effects, although individual patients have occasionally complained of augmentation effects that were alleviated by increased dosing (Earley and Allen 1996; Earley, Yaffee et al.

1998). Additionally, treatment must often be supplemented with domperidone (an extracerebral D2 antagonist) in order to prevent side effects such as nausea and orthostatic hypertension. More recently, newer primary dopamine agonists have proven effective (Montplaisir, Nicolas et al. 1999; Ondo 1999). Consistent with the clinical observations, 3 of 4 brain imaging studies of RLS patients have shown striatal dopamine abnormalities (Staedt, Stoppe et al. 1995; Turjanski, Lees et al. 1999; Ruottinen, Partinen et al. 2000; Eisensehr, Wetter et al. 2001).

In addition to dopamine agonists and antagonists, opioids, benzodiazepines, carbamazepine, valproic acid, gabapentin, clonidine, iron and magnesium have all been reported to be beneficial, but either experience with these drugs is limited or the treatment effects are not convincing (Stiasny, Wetter et al. 2000). Non-pharmacologic treatments include improved nutrition, exercise, and sleep maintenance. Additionally, many patients tend to find that engaging themselves in a mental activity requiring concentration, such as painting or computer programming, alleviates symptoms. Caffeine, antihistamines, and cold preparations have been reported to aggravate symptoms, while alcohol may offer a brief reduction in symptoms but with rebound worsening of symptoms.

While much of the clinical intervention for RLS has focused on the dopaminergic system, there is a growing body of literature that suggests a significant role for iron. Iron deficiency has been recognized as a significant contributing cause of RLS for almost 50 years (Nordlander 1953; Ekbom 1960). High-dose, intravenous iron therapy brought about complete relief of symptoms in 21 out of 22 RLS patients for periods of up to 3-9 months, even though the majority had normal iron status prior to treatment (Nordlander 1953). Additionally, oral iron supplementation has been shown to improve symptoms in some RLS patients (O'Keeffe, Gavin et al. 1994). Pregnancy is an example of a secondary cause of RLS that supports the causative

role of iron deficiency in RLS, as there is an increased incidence of RLS in pregnancy attributable to iron deficiency (Ekbom 1960). A negative correlation between serum ferritin levels (ferritin is the storage form of iron in the body) and RLS symptom severity has been noted in two studies where decreasing ferritin is associated with increasing RLS severity (O'Keeffe, Gavin et al. 1994; Sun, Chen et al. 1998). Cerebrospinal fluid (CSF) levels of ferritin, iron, and transferrin support the theory that brain iron is deficient in RLS. A 65% decrease in CSF ferritin and a three-fold increase in CSF transferrin was reported in RLS patients compared to controls, despite normal serum ferritin and transferrin in both populations (Earley, Connor et al. 2000). An elevation in transferrin coupled with a decrease in ferritin is a common observation in the serum of iron-deficient individuals. The existence of a similar relationship in the CSF for transferrin and ferritin would represent convincing evidence for a brain iron deficiency. This possibility is investigated and explored in detail in Chapter Two.

Direct evidence of brain iron deficiency in RLS comes from an MRI analysis that indicates brain iron levels in the substantia nigra are below normal in individuals with RLS (Allen, Barker et al. 2001). Both the substantia nigra and the putamen showed iron losses in proportion to RLS severity. A recent histopathological analysis also provides support that the substantia nigra in RLS is iron-deficient (Connor, Boyer et al. 2003). This latter analysis also noted that no histopathological alterations were observed in the substantia nigra that were unique to brains of individuals who had RLS. Thus, the conclusion was that RLS is a functional disorder involving a disruption in brain iron acquisition by the substantia nigra or perhaps specifically the neuromelanin cells in this region.

A definitive animal model has not been established for RLS, but some promising avenues have been opened. Belgrade rats have a defect in an iron transport protein that results in chronic,

global iron deficiency (Fleming, Romano et al. 1998; Burdo, Menzies et al. 2001; Zywicke, van Gelderen et al. 2002). Although a similar defect has not been demonstrated in RLS patients, these rats may provide insights into how the brain responds to chronic iron deficiency and how iron supplements may influence brain iron status. Given that some RLS patients respond to dietary iron supplementation (Ekbom 1960; Davis, Rajput et al. 2000) and more to intravenous iron, it is likely that understanding brain iron transport will reveal insights into the pathophysiology of RLS. Additionally, studies on iron-deficient rats by Beard et al have shown ventral midbrain iron concentration and dopamine D1 receptor density to be associated with exploration and repeated movements, respectively (Beard, Erikson et al. 2002). Iron is a cofactor for tyrosine hydroxylase, which is in turn the rate-limiting enzyme for dopamine metabolism (Cooper, Bloom et al. 1991). Iron chelators were effective in reducing the content of dopamine and its metabolites, as well as dopamine turnover, in rats (Ward, Dexter et al. 1995). Therefore, a decrease in iron may decrease dopamine synthesis and thus dopamine availability. For that reason, the iron-deficient rat model studied by Beard et al reveals the potential relationship between brain iron status and the dopaminergic system that appears to be affected in RLS (Beard, Erikson et al. 2002). A third animal model of promise in the study of RLS is the developmental iron-deficient rat, as described earlier in this chapter. We utilized this rat model to study possible iron-related predisposing factors for RLS, as discussed extensively in Chapter Four.

The extensive interactions of iron in the body and brain – and the diseases that result from the seemingly small perturbations of iron balance – were the impetus for the work in the following chapters. Macroscopically, it is of little surprise that iron plays such a fundamental role in health and disease. The presence of iron predates our own existence by billions of years;

its age-old production within massive stars is even used as a marker of age in the universe. In more recent planetary time, our Earth was created with its own iron core, with the crust on which we stand being the byproduct of planetary smelting. The omnipresence of iron universally and locally has undeniable implications for our biology: we cannot attain health without iron balance, as evidenced by the scope and number of diseases resulting from defective iron regulation. Accordingly, a full understanding of the intricate complexity of iron transport into and throughout the human body will have tremendously broad implications.

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Chapter 2

Analysis of Ferritin, the Ferritin Subunits, and Transferrin in CSF in Restless Legs Syndrome

Abstract

Restless Legs Syndrome (RLS) is a neurological disorder that may be related to iron misregulation at the level of the central nervous system. Evidence that iron is involved in RLS comes from MRI data, autopsy studies, analyses of cerebrospinal fluid, and correlations of symptoms with serum ferritin. To further examine the possibility that brain iron status is insufficient in RLS, we determined ferritin levels in the cerebrospinal fluid. Specifically, we measured whole molecule ferritin and also differentiated between the H- and L- subunits of ferritin, because these peptides are expressed from different chromosomes and have different functions. We measured ferritin and H- and L-ferritin subunit levels in control and RLS human cerebrospinal fluid (CSF) using immunoblot analysis and found that both ferritin and the H- and L-ferritin subunits are significantly decreased in early but not late-onset RLS. Transferrin levels were not significantly changed between groups. Additionally, we quantified total protein in each CSF sample to establish that the decrease in ferritin subunits in RLS did not reflect a decrease in total protein in CSF. Further, we used equal amounts of total CSF protein in the immunoblot analyses, in contrast to previously published studies that provided only volumetric data, to determine which approach was more accurate for quantifying the amount of ferritin relative to other proteins in CSF. Our results establish a protein standard in RLS, provide a comparative analysis of protein-controlled vs. volumetric immunoblot techniques, and argue for a profound loss of iron storage capacity in the brain in RLS, specifically in the early-onset RLS phenotype.

These data suggest that CSF ferritin levels may provide a biomarker for assisting in the diagnosis of RLS.

Introduction

Restless Legs Syndrome (RLS) is a disorder of the sensorimotor system characterized by an irresistible desire to move the extremities, with the legs generally affected to a greater degree than the arms (Earley, Allen et al. 2000). The disease has a significant impact on the quality of life of its sufferers (Abetz, Allen et al. 2004), underscoring the importance of understanding its pathology and elucidating effective treatment. Among the most frequently considered causes of the syndrome is an insufficient amount of brain iron. The role of low brain iron status in RLS pathology has been implicated by several different investigational methods, including magnetic resonance imaging (MRI) (Allen, Barker et al. 2003), CSF analysis (Earley, Connor et al. 2000), and autopsy (Connor, Boyer et al. 2003). Low brain iron storage in turn may result in reduced dopamine synthesis and/or receptor function (Youdim, Ben-Shachar et al. 1989; Chen, Connor et al. 1995; Nelson, Erikson et al. 1997). Further evidence supporting the role of brain iron dysfunction in RLS comes from a study by Earley et al (Earley, Heckler et al. 2004) in which intravenous iron dextran significantly improved mean global RLS symptom severity, total sleep time, and hours with RLS symptoms. MRI analysis supported these findings, showing increased iron in substantia nigra and prefrontal cortex two weeks post-treatment.

In this study, we focused on the analysis of ferritin in the cerebrospinal fluid. Ferritin plays a primary role in both iron storage and detoxification and is found both intracellularly and in a secreted form in the blood (Bellotti V 1987). Ferritin molecules are composed of two subunit types, H and L. A third, distinct plasma ferritin has also been proposed to exist, having a

distinct amino acid sequence from intracellular L and H ferritins (Linder, Schaffer et al. 1996; Tran, Eubanks et al. 1997). Generally, H-chains are important in ferrous iron oxidation and often increase in response to acute inflammation. H-ferritin may serve as a regulator of cellular differentiation as well as a cytoprotectant (Chou CC 1986; Chazenbalk GD 1990; Chazenbalk GD 1990; Broxmeyer HE 1991; Balla G 1992; Cozzi A 2003). L-ferritin levels are often reflective of long-term iron storage, and are involved in core formation of the mature 24-mer protein (Levi S 1994). These diverse subunit roles highlight the importance of understanding ferritin composition in normal and disease states. Subunit composition of the mature ferritin varies greatly among mammalian tissues, with L-rich ferritins generally being found in iron storage organs such as liver and spleen, and H-rich ferritins being located more in organs such as heart and brain (Harrison and Arosio). In the brain, H-ferritin predominates (Sanyal B 1996), but the ratio of H to L subunits is cell specific. Neurons express almost entirely H-ferritin and microglia express predominantly L-ferritin (Connor 1994; Han J 2002). Thus far, only a few studies have investigated the ferritin H- and L-subunits in disease states in any body tissue. One such study looked at the relative amounts of H- and L-ferritin in liver, spleen, and adrenal gland from Niemann-Pick disease Type C (NPC) patients and found both ferritin subunits to be deficient in all tissue specimens of NPC patients (Christomanou, Vanier et al. 2000). To our knowledge, no studies have specifically investigated H- and L-ferritin in the CSF in RLS. Previous RLS CSF studies revealed that whole molecule ferritin was lower in RLS compared to controls, and that there was a strong correlation between the age of symptoms onset and the CSF ferritin values, such that the earlier the age of onset, the lower the ferritin. Further, the early-onset (less than 45 years of age) RLS group had significantly lowered CSF whole molecule ferritin compared to controls. Given this knowledge, we undertook this study to determine if one

or both of the ferritin subunits were changed in RLS compared to controls, and ascertain if significant differences existed between the RLS age-of-onset subtypes.

Materials and Methods

Patient Characteristics – This is an IRB-approved study. The research was carried out according to the principles of the Declaration of Helsinki. Informed consent was obtained from all participants. We used CSF collected from a total of 39 individuals at Johns Hopkins University, 25 who were diagnosed with RLS (12 early-onset RLS, 13 late-onset RLS) and 14 control subjects who did not have RLS. The lumbar punctures (LP) were all performed at 10 PM in the evening at a time when symptoms are maximal. The patients fit the standard characteristics described previously (Earley 2005) and briefly reported here. To be eligible, subjects with idiopathic RLS had to meet all 4 criteria for the syndrome (Allen, Picchietti et al. 2003); report nightly symptoms prior to treatment for at least six months; show a positive clinical response to dopaminergic agents; and have PLMS >20/hr when off of medications. Patients developing RLS in relationship to other specific causes like anemia, iron deficiency, renal dialysis, peripheral neuropathy, neurodegenerative disorder or other obvious medical problems or medications were excluded from involvement in the study. In order for control subjects to be eligible, they could have no symptoms to suggest RLS; no first degree relatives with RLS; and PLMS <15/hr (on screening or on PSG). To rule out RLS in living, first-degree relatives of control, telephone interviews were performed using methods previously described (Hening, Allen et al. 2003). Control subjects were matched to RLS subjects for age- (± 5 years), race- and gender. General exclusion criteria that applied to both RLS and controls subject were the presence of sleep apneic/hypopneic rates of >30/hr, serum ferritin <18 μg/l; percent iron

saturation <16%; medical or surgical factors that might limit the performance or interpretation of the investigations to be performed in this study. RLS and psychoactive medications (except nicotine and caffeine) as well as any supplements with iron were stopped 2 weeks or 6-half-lifes (which ever was the longest) prior to admission to the GCRC for the study.

H & L Ferritin - The human recombinant ferritins utilized in this study [recombinant heavy (rH) and recombinant light (rL) chain ferritins], as well as the rH02 and LF03 antibodies, were the generous gift of Paolo Arosio (Milan, Italy).

Protein Measurement - The CSF protein levels were determined using the Pierce Micro BCA Assay for Protein Determination (Pierce #23235).

Immunoblot Analysis - Immunoblot analysis was performed using the Schleicher & Schuell Minifold II apparatus (Keene, NH) with a nitrocellulose membrane (Schleicher & Schuell). For the transferrin studies, apo-transferrin (Sigma, T-1147) was used as a standard, with 1:2500 goat anti-human Tf (E-Y Laboratories, San Mateo CA) primary antibody and 1:12000 rabbit anti-goat IgG coupled to HRP (Sigma) secondary antibody. For whole-molecule ferritin, the standard was ferritin type V: from human spleen (Sigma, F-6879), with 1:1000 rabbit Anti-Human Ferritin (Sigma, F-5012) primary antibody, and the secondary antibody anti-rabbit IgG (whole molecule) peroxidase conjugate (Sigma, A-9169) diluted 1:6000. The primary antibody concentrations for both H and L ferritin were 1:1500. The secondary antibody in the H- and L-ferritin subunit studies was anti-mouse IgG (whole molecule) peroxidase conjugate developed in rabbit (Sigma A-9044) used at a concentration of 1:5000.

The antibodies for all immunoblots were constituted in 40 ml of 5% nonfat dried milk in TBS-Tween. Each immunoblot included a standard curve, and every sample on the immunoblot was within the linear range of the standard curve. Each blot also contained a pooled CSF sample in triplicate. Densitometry was performe don each immunoblot, and an equation was generated from the linear regression anlysis of the standard curve. Data from all blots in an analysis set was then normalized utilizing the pooled CSF sample on each blot. Each sample on all immunoblots was applied at two concentrations (20µg/ml and 40µg/ml for whole molecule ferritin and transferrin; 10µg/ml and 20µg/ml, for H- and L ferritin) in triplicate. After the completion of all experiments in a set, the concentration with the greatest linear range was chosen for analysis of all immunoblots in that set. A volume of 100µl was added to each well to arrive at the final concentrations.

Briefly, the protocol was as follows: While in the Minifold II apparatus, the nitrocellulose membrane was pre-rinsed twice with TBS, the samples were applied (100ul solution for samples, 200ul solution for standards), and the membrane was rinsed a final time with TBS. The membrane was then immediately removed and placed in 5% nonfat dried milk in TBS for 1hr. The membrane was rinsed 3 times (5 minutes each) with TBS at this point, and between each of the following incubation steps. The membrane was then incubated with primary antibody for one hour, rinsed, and then incubated with secondary antibody for one hour. A final rinse was performed with TBS-Tween. All procedures were performed at room temperature. The membrane was then developed for visualization using Western Lightning chemiluminescence system (PerkinElmer, Wellesley, MA), and the blots were exposed to film. Immunoblot film analysis was performed using a laser densitometer system (100 A, Molecular Dynamics,

Sunnyvale CA with Quantity One Software, Biorad). The average peak optical density for the triplicate samples was calculated.

All quantitative data are expressed as mean ± standard error. Statistical comparisons were made using the student's t-test, variance, and Fisher's PLSD.

Results

The ferritin and transferrin results are compared to the results from a previous study by our group in Table 2.1. The previous study was done on a small population with CSF sampling at 10 AM, in contrast to the 10 PM sampling done for this study. Whole-molecule CSF ferritin was significantly decreased in RLS compared to controls ($p < 0.025$). Within the RLS group, CSF Ferritin was significantly lower compared to controls for early-onset RLS ($p < 0.003$) but not late-onset RLS ($p > 0.3$). The difference between early-onset and late-onset RLS was marginally non-significant ($p = 0.062$). Additionally, the age at which symptoms were reported to have first occurred showed a significant correlation to CSF ferritin (Figure 2.1, $r = +0.64$, $p < 0.0002$). This correlation between age-of-onset and CSF ferritin was significant for early-onset ($r = +0.65$, $p < 0.01$) but not late-onset RLS groups ($p = 0.29$). Transferrin was not significantly different between control and RLS CSF samples (Figure 2.2).

The specificity of the H- and L-monoclonal antibodies is shown in Figure 2.3. Human spleen ferritin was used as a control for the primary antibodies on both blots, because it contains both H- and L-ferritins, in a ratio of approximately 10-20% H-ferritin and 80-90% L-ferritin (Powell, Alpert et al. 1975; Arosio, Adelman et al. 1978; Stefanini, Chiancone et al. 1982; Harrison and Arosio 1996; Grady, Zang et al. 2002).

The concentrations of the H- and L-ferritin subunits were determined and are reported per unit of total protein (Figure 2.4A). The concentrations of both H- and L-ferritin levels are significantly decreased ($p<0.05$ and $p<0.002$, respectively) in the CSF of RLS patient samples compared to the control samples. The percentage decrease was 50% for H-ferritin and 116% for L-ferritin. Because clinical measurements of CSF are routinely performed volumetrically, the amounts of the ferritin subunits were calculated per volume of CSF. Using total volume instead of total protein as the basis for the ferritin concentrations, a significant decrease in both H- and L-ferritin is still observed in the RLS patients (Figure 2.4B). The distribution of the data around the mean, however, was greater in the volumetric analysis ($p<0.05$). Further assessment found that the early-onset but not the late-onset RLS group had significantly lowered CSF H- and L-ferritin compared to controls (Figure 2.5). For additional detailed immunoblot results for H- and L ferritin, please see the Appendix, which displays the data generated from each standard curve, as well as the data resulting from normalization of the pooled samples from all the blots.

To determine that the decrease in ferritin and ferritin subunits did not simply reflect a loss of total protein, we determined the total protein levels in the CSF (Figure 2.6). This analysis revealed that there is no significant difference between the mean concentration of total CSF protein in RLS compared to control (718 µg/ml ± 30.01 and 659 µg/ml ± 83.75, respectively).

Discussion

In this study, we did not show an increase in CSF transferrin in RLS, as previously noted in a much smaller, earlier study (Earley, Connor et al. 2000). There are several differences, however, between the two studies (Table 2.1). The most distinctive difference between the 2 studies is the time at which samples were collected: 10 AM versus 10 PM. There are known

circadian changes in serum iron regulation, with the primary change being a drop in serum iron levels of about 50% at night (Ahluwalia, Lammi-Keefe et al. 1993). The drop in serum iron at night could have a consequence on brain iron status at night and thus have an effect on symptoms. We are not aware of any other studies that have examined brain or CSF for circadian changes in iron regulation, with this being the first study to even evaluate subjects at night. There was little difference between the 10 AM and 10 PM control samples, and therefore there appears to be little diurnal changes in CSF iron regulation under normal conditions. The RLS group, in contrast, showed differences for both CSF ferritin and transferrin between the 2 samples, suggesting a distinct diurnal variation in CSF iron regulation in RLS. It is possible that some of the diurnal differences in the RLS-CSF ferritin and transferrin may reflect differences in the antibodies, as each study utilized different transferrin and ferrittin antibodies. Additionally, the second study controlled for total protein in the CSF. Minimally, the previous morning samples appear to reflect the time at which differences between RLS and control values for both ferritin and transferrin are most pronounced.

We have established that whole-molecule ferritin, H-ferritin, and L-ferritin are decreased in early-onset RLS. These data are consistent with previous reports (Earley, Connor et al. 2000) and extend the findings to the individual ferritin subunits. The data provide further support for the notion that RLS is a disorder related to insufficient iron at the level of the central nervous system (CNS) (Earley, Connor et al. 2000; Connor, Wang et al. 2004). We also established that total protein amounts in RLS CSF are normal — an important piece of information in elucidating the pathology of RLS. Changes in total CSF protein occur in a number of neurological disorders. For example, the presence of tumors, multiple sclerosis, diabetes, hemorrhage, polyneuritis, trauma, and blood in the CSF have all been associated with an increase in total CSF protein,

while a decrease in total CSF protein could result from any condition causing rapid CSF production (Reiber 2003). Additionally, changes in the CSF flow rate could lead to reduced volume exchange with increased protein influx into the CSF due to unchanged permeability coefficients (Ohman 1994). Given the high incidence of secondary RLS resulting from iron deficiency and renal dialysis, it was important to determine if protein concentration could influence any future diagnostic use of ferritin levels in the CSF for RLS. Furthermore, although the mean protein values are not significantly different between RLS and control, there is a considerably greater range of protein values in the control group (Variance significant at 95% confidence interval). The meaning of the relatively low variance in the RLS group is not known, partly because so little is known about the origin of proteins in the CSF. Regardless of the total protein variance, the concentrations of the ferritin subunits in the CSF were tightly grouped indicating that the range of protein concentration in this case is not a confounding variable. The lumbar punctures all occurred at 10pm, so the variance is not due to sampling from different time points of a circadian rhythm.

The origin of ferritin in the CSF has not been determined. The interpretation of the decrease in CSF ferritin is that it reflects a decrease in brain iron status. As mentioned in the introduction, elevated ferritin in the CSF is associated with inflammatory states (Scaccabarozzi, Arosio et al. 2000). The choroid plexus contains high levels of proteins involved in iron uptake and mobilization, including ferritin. Ferroportin, a protein involved in export of iron from cells, has been demonstrated in choroid plexus (Connor, Menzies et al. 2001; Wu, Leenders et al. 2004) and the mRNA for transferrin, the iron mobilization protein, is expressed in relatively high abundance in rodent choroid plexus (Fenstrmacher 1966). Transferrin is thought to be actively released from choroid plexus because of its relatively high concentration compared to that in the

serum (Benkovic and Connor 1993). Release of ferritin from cells such as hepatocytes and macrophages has been reported (Custer, Balcerzak et al. 1982; Brock, Esparza et al. 1984; Alvarez-Hernandez, Felstein et al. 1986; Saito, Nishisato et al. 1986; Beguin, Huebers et al. 1989; Bezkorovainy 1989; Cable, Connor et al. 1998; Cheepsunthorn, Radov et al. 2001), and a ferritin receptor in the brain has been reported (Hulet, Heyliger et al. 2000; Hulet, Menzies et al. 2002), so it is possible that ferritin is released by choroid plexus or the ependymal cells lining the ventricles. These data indicate that further studies are warranted to determine the source of ferritin in the CSF and its relationship to brain iron status.

Although there has been little previous investigation of H- and L- ferritin subunit levels in human CSF in any neurological syndromes or diseases, several studies have looked at whole ferritin levels in disease. Many of these studies focus specifically on neurological diseases or on the neurological implications of systemic diseases. Levine et al determined the levels of whole ferritin in Multiple Sclerosis (MS) and demonstrated elevated CSF ferritin levels in active chronic progressive MS patients compared to normals (LeVine, Lynch et al. 1999). In a study of Alzheimer's disease (AD), Parkinson's disease (PD), and multiple system atrophy (MSA), CSF ferritin was found to be significantly elevated in AD patients compared to PD and MSA patients as well as controls (Kuiper, Mulder et al. 1994). A study of subacute sclerosing panencephalitis (SSPE), correlated CSF ferritin, creatine kinase, and neopterin levels to clinical symptoms and MRI, and found that all three substances appear to be biochemical markers for the extent of the lesions in SSPE (Murata, Hattori et al. 1992) . A large scale analysis of ferritin in CSF by found that of 470 samples, high ferritin was found in infectious meningoencephalitis, in vascular diseases of the CNS, and in cases of dementia without vascular cause (Sindic, Collet-Cassart et al. 1981). Elevated CSF ferritin is also a non-specific finding in HIV patients suffering acute

neurological episodes (some of the diagnoses included crytocococcal meningitis, CNS lymphoma, and herpes simplex or cytomegalovirus encephalitis) (Deisenhammer, Miller et al. 1997). Whole molecule ferritin CSF levels have also been studied in the context of cancer with CNS involvement. Sato et al found CSF ferritin concentrations to be high in patients with glioblastoma (103 ng/ml) compared to patients with meningitis or headache, at levels of 5.4 and 4.3 ng/ml, respectively (Sato, Honda et al. 1998). Another study investigated CSF ferritin in four groups of patients: patients with malignant disease, both with and without CNS involvement, and non-cancer patients with inflammatory as well as noninflammatory neurologic disorders. They found CSF ferritin to be increased in the majority of patients with inflammatory neurologic disease and in patients with malignant involvement of the CNS, but not in patients with noninflammatory neurologic disorders or malignant disease without CNS involvement (Zandman-Goddard, Matzner et al. 1986). Total ferritin is reportedly decreased in CSF of individuals with RLS (Earley, Connor et al. 2000; Mizuno, Mihara et al. 2005). The studies described above illustrate the role of whole ferritin in a broad range of diseases, implicating its roles in inflammation, oxidative stress, and cancer, all in the context of the brain. Given what is already known about the functional differences between H- and L-ferritin, analysis of the subunit ferritins would be helpful in elucidating pathology more clearly by discerning between acute and chronic iron dysfunction that may lead to insights on brain iron storage and the brain's response to inflammation.

Previous studies found a significant decrease in H-ferritin staining in RLS brains compared to controls, though L-ferritin staining was largely unchanged (Connor, Boyer et al. 2003). As discussed by Connor et al (Connor, Boyer et al. 2003), it is possible that the L-ferritin staining in the autopsy studies may reflect a redistribution of iron from the neurons into the glial

cells, because most of the L-ferritin staining was associated with microglia and astrocytes. Thus, our current findings support the concept that the CSF reflects an iron-deficient state for the brain, but the ferritin concentrations measured from brain homogenates reflect a more complex interaction among the various cell types that includes compensatory responses. Very little is known about iron transport between cells in the brain.

The observation that early-onset RLS is associated with significant decreases in both H- and L-ferritin CSF, but late-onset RLS CSF values are not significantly decreased, suggests that age-of-symptom-onset is a relevant phenotype of RLS. The current finding provides further support of our previous work in whole molecule ferritin that also found that early-onset but not late-onset RLS had lower CSF whole-molecule ferritin compared to controls (Earley 2005). These distinct phenotypes may define two distinct disease processes (Polydefkis, Allen et al. 2000; Allen, La Buda et al. 2002; Winkelmann, Muller-Myhsok et al. 2002). The results of the present study establish that total protein is not affected in the CSF in RLS, provide a comparative analysis of protein-controlled vs. volumetric immunoblot evaluative assays, and argue for a profound loss of iron storage capacity in the brain in RLS. The differences in the H:L ferritin subunit ratios in the CNS can be informative regarding acute versus chronic changes in iron availability and usage. In the case of RLS, both subunits are decreased in early-onset RLS relative to normal in the CSF, suggesting a chronic, active iron insufficiency. The decreases in whole molecule ferritin and L- and H-ferritin in RLS may serve as biomarkers for the disease and disease-onset subtypes.

Table 2.1. A comparison of CSF ferritin, transferrin, and iron measures based on differences in sample collection time is displayed in Table 1 (* $p<0.001$ for AM compared to PM). The AM measurements are from previous work done by our group.

	Time	Ferritin (mg/L) (whole molecule)	Transferrin (mcg/L)	Iron (mcg/L)
Control	AM	3.50 ± 1.6	6.71 ± 4.3	18.0 ± 16.8
	PM	2.89 ± 0.9	5.17 ± 1.8	26.2 ± 19.1
RLS	AM	1.11 ± 1.0*	26.4 ± 19.7*	25.3 ± 5.0
	PM	2.43 ± 0.7	4.9 ± 1.9	27.4 ± 16.6

* $p<0.001$ for AM compared to PM

Table 2.1

Figure 2.1. **Relationship between CSF ferritin and age of symptom onset.** The correlation between the current measured CSF ferritin and the reported age at which symptoms started is shown in Figure 2 ($r = 0.64$, $p < 0.0002$). Generally, the earlier the age at which symptoms began, the lower the current CSF ferritin level.

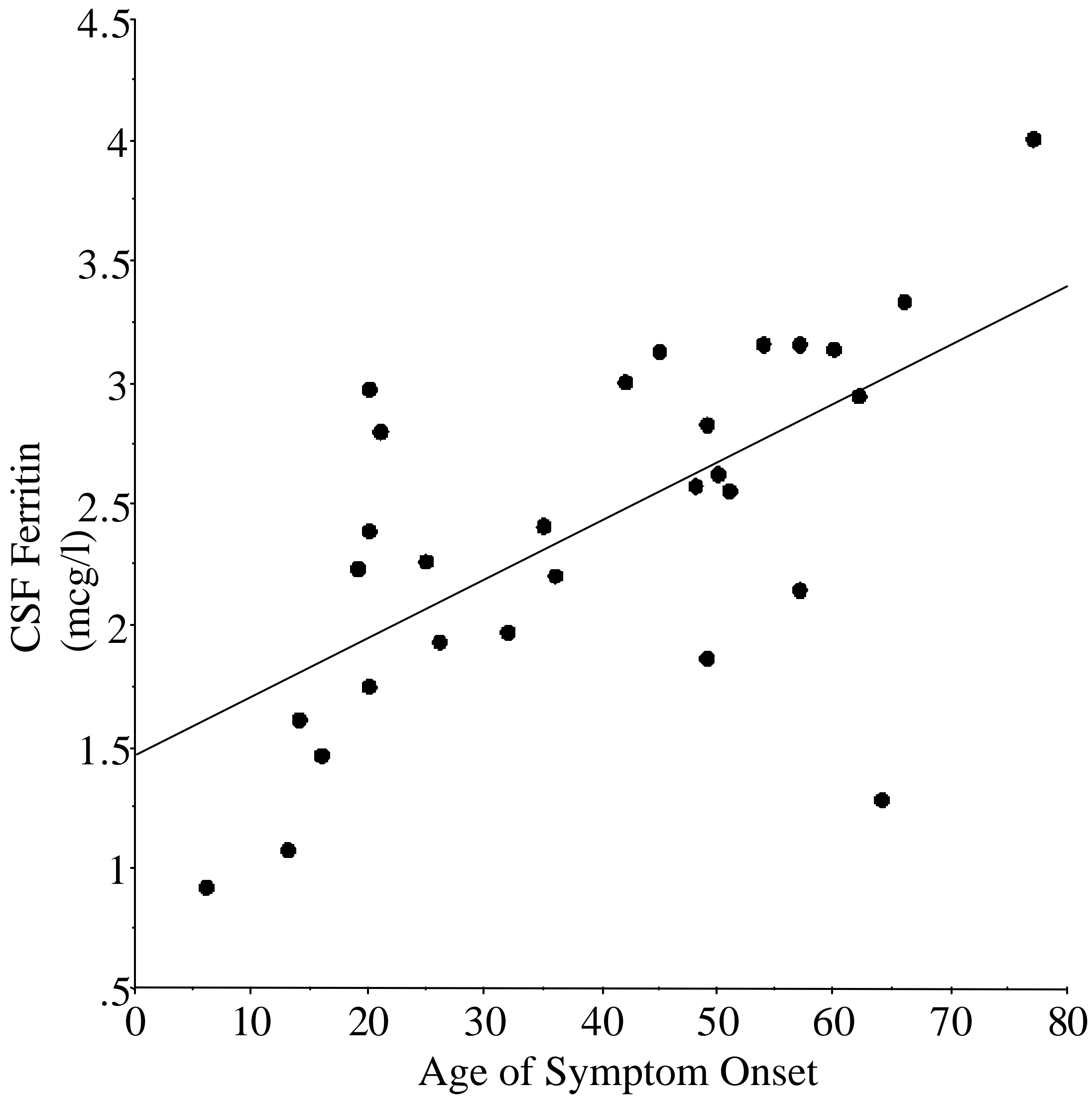


Figure 2.1

Figure 2.2. Transferrin CSF levels in control and RLS. Transferrin levels were not significantly changed between control samples when compared to RLS patient samples (5.17 mg/dl ± SEM 1.8 and 4.9 mg/dl ± SEM 1.9, respectively). The data were analyzed per unit of total protein in the CSF. Error bars display the SEM.

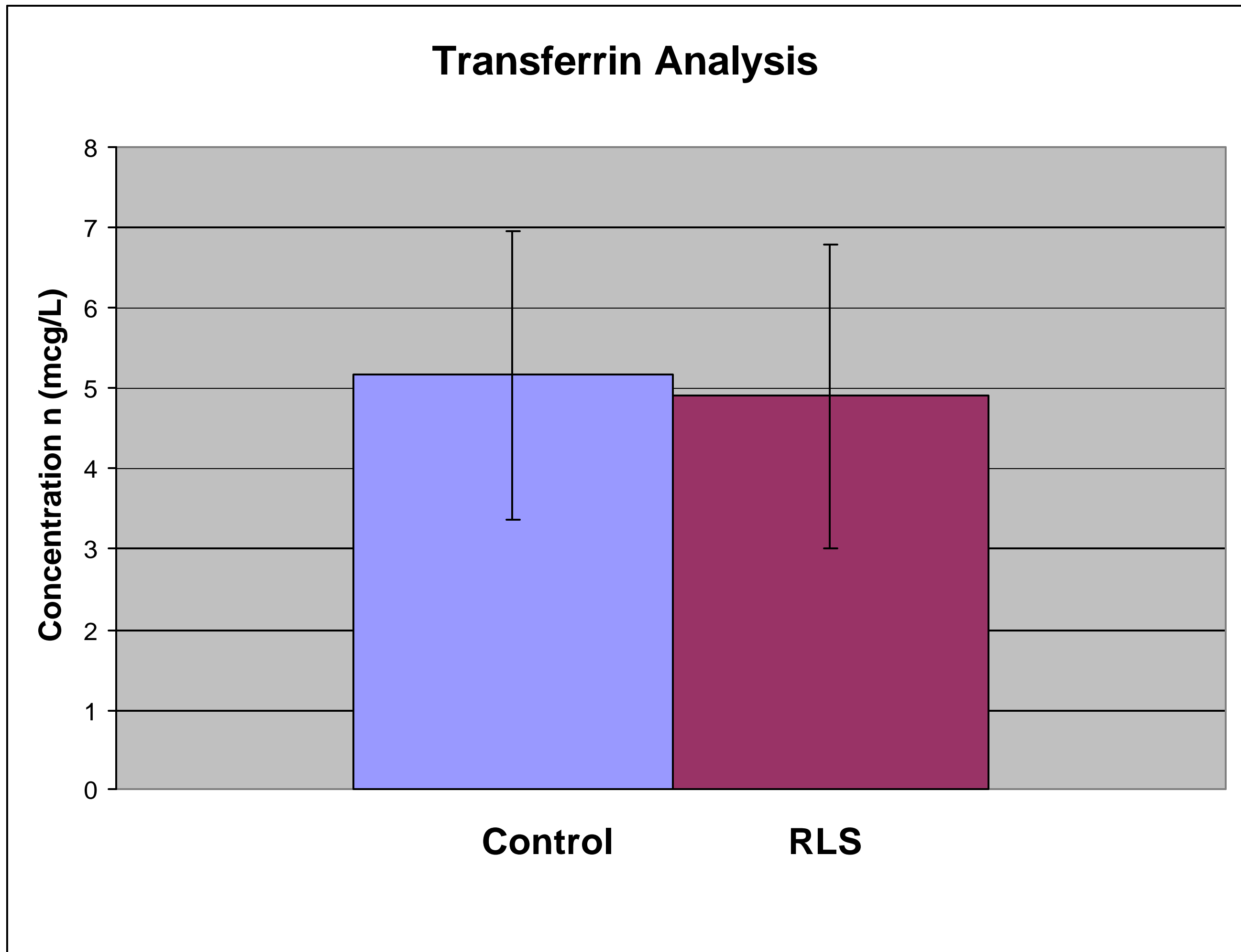


Figure 2.2

Figure 2.3. Representative monoclonal ferritin immunoblots. Two representative slot blots are shown in this figure. The left blot utilized the monoclonal H-ferritin antibody, and shows no detection of L-ferritin at any concentration. Conversely, the blot on the right demonstrates the lack of detection of H-ferritin with the monoclonal L-ferritin primary antibody. Human spleen ferritin was used as a control for the primary antibodies in both blots, as it is known to contain both H- and L- ferritins. The representative CSF samples demonstrated the presence of both H- and L-ferritin, as did the Pooled Sample (comprised of 5 control individuals).

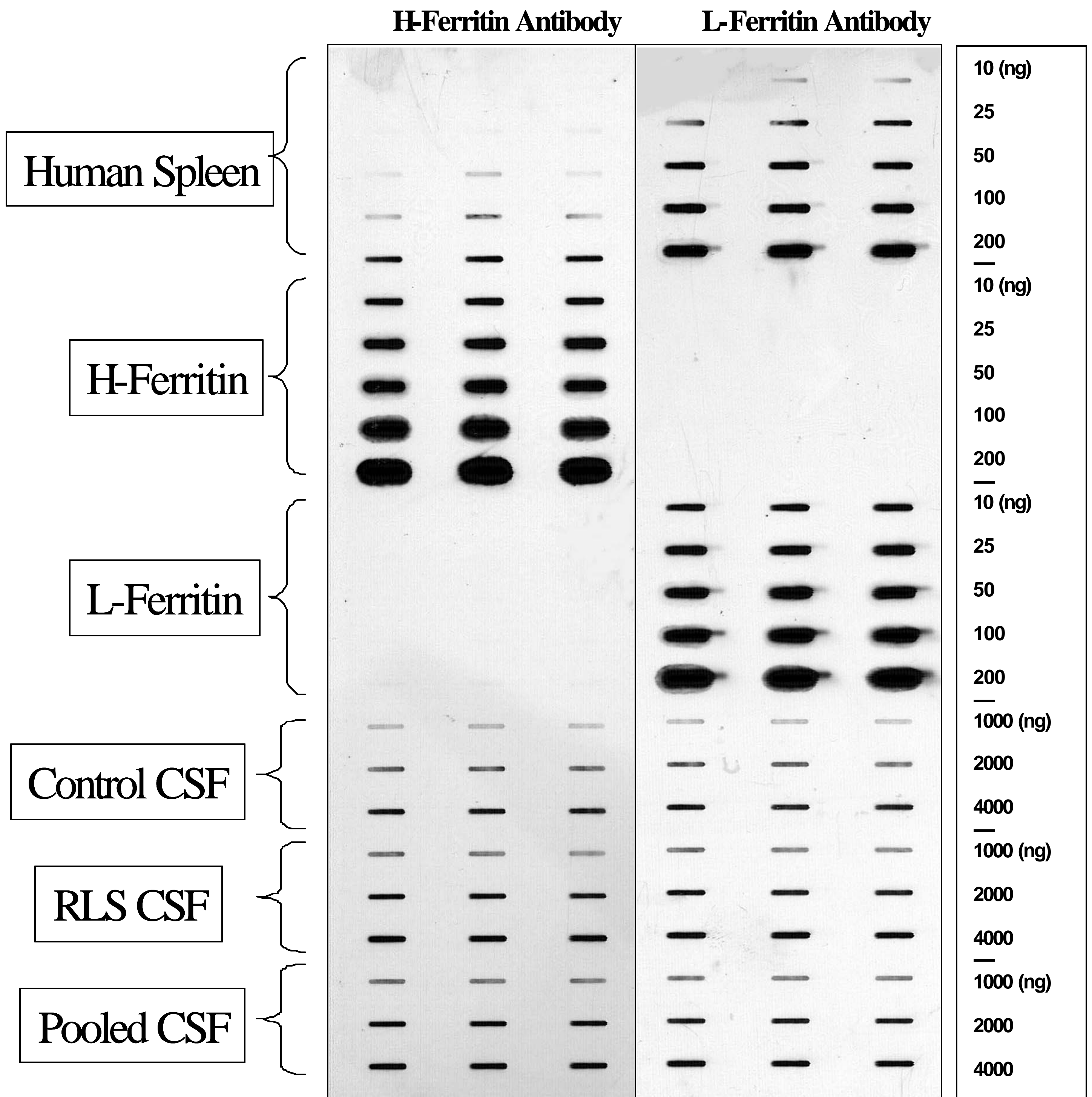
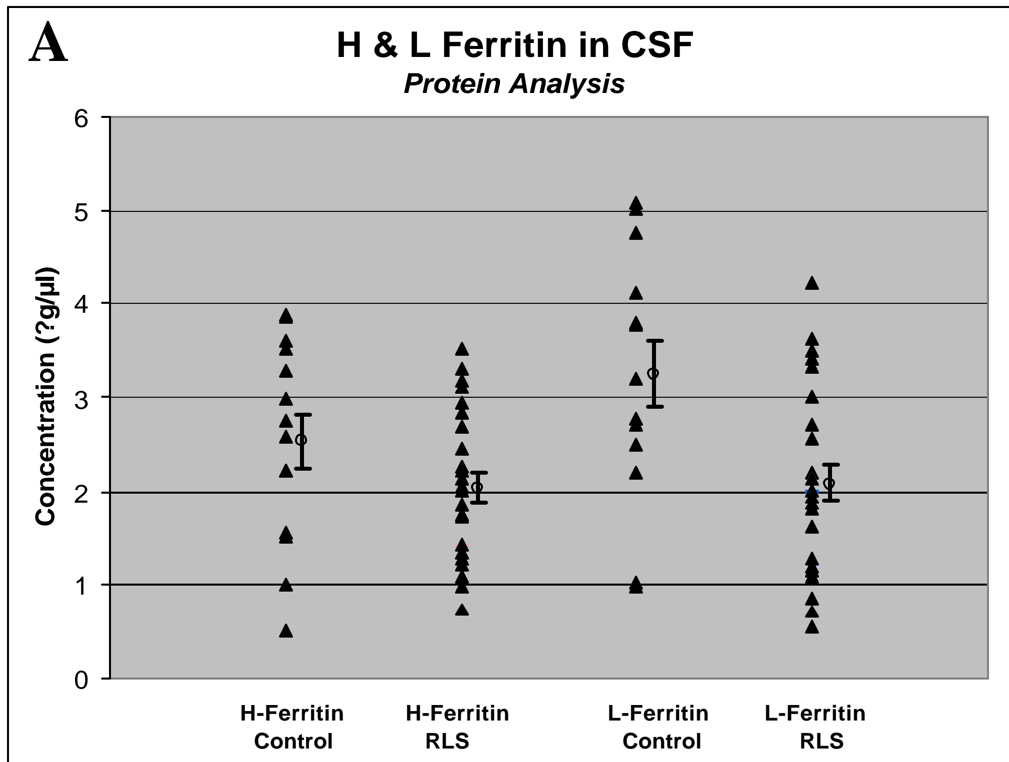


Figure 2.3

Figure 2.4. Levels of H- and L-ferritin in CSF of RLS samples compared to control samples. H- and L-ferritin levels were both significantly decreased ($p<0.05$ and 0.002, respectively) in the CSF of RLS patient samples compared to the control samples using unit protein as the standard (A). A significant decrease in both H- and L-ferritin levels in CSF from RLS patients is observed also when the data are considered per unit volume ($p<0.04$ and 0.02, respectively) without consideration of total CSF protein (B). The volume of ferritin subunits in the CSF was derived from the protein analysis. There are fewer outliers in the analysis when the data are considered per unit protein as opposed to volumetrically. The SEM is displayed beside each set of data points (*$p<0.05$ and ** $p<0.005$).



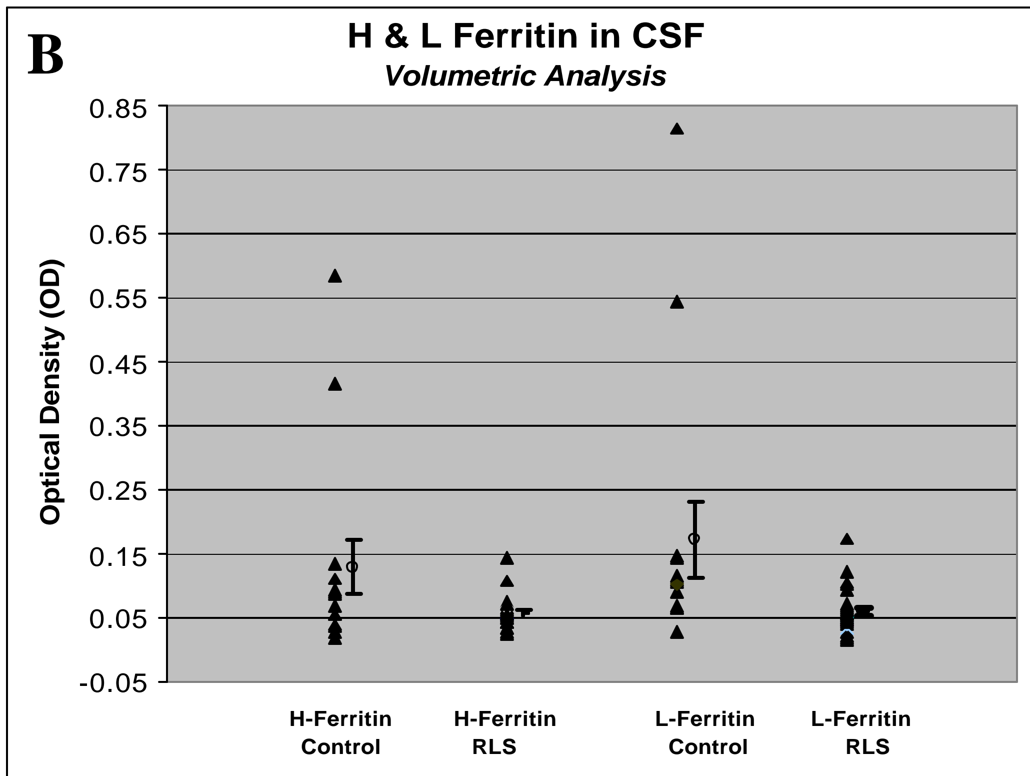
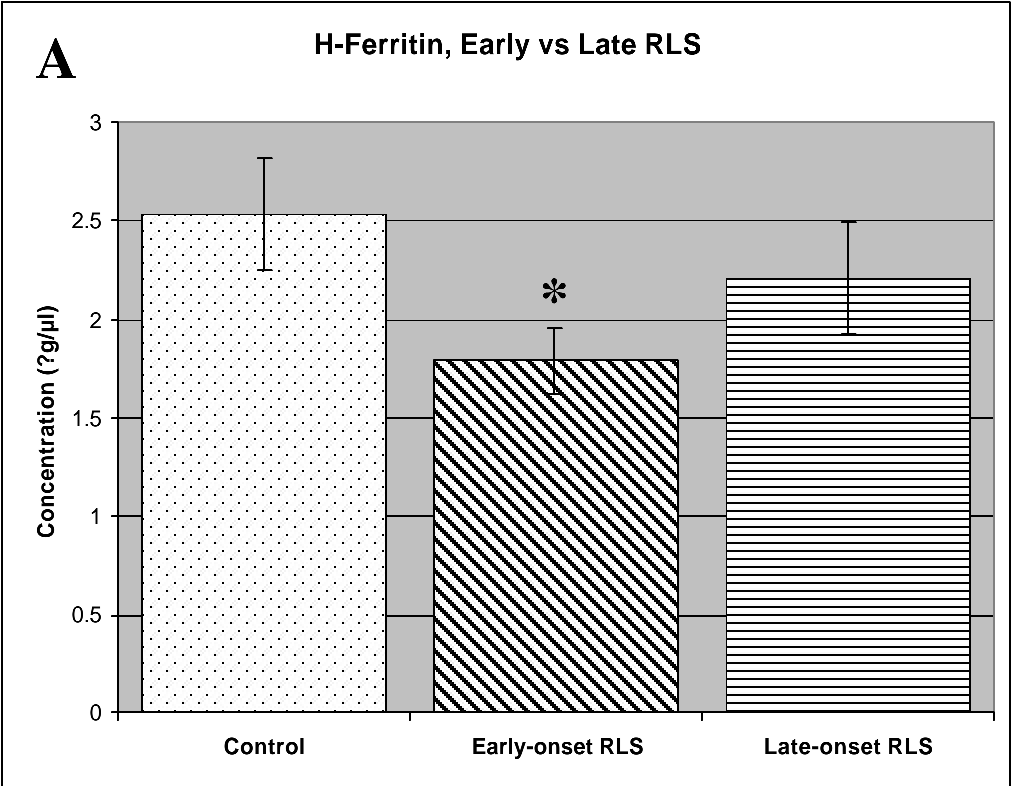


Figure 2.4

Figure 2.5. H- and L-ferritin levels in early- and late-onset RLS samples compared to controls. H-ferritin (A) and L-ferritin (B) levels were significantly decreased in early-onset RLS patient samples but not in late-onset RLS patients when compared to controls. The data were analyzed per unit of total protein in the CSF. Fisher's PLSD, p-value *<0.05 and ** <0.005. Error bars display the SEM.



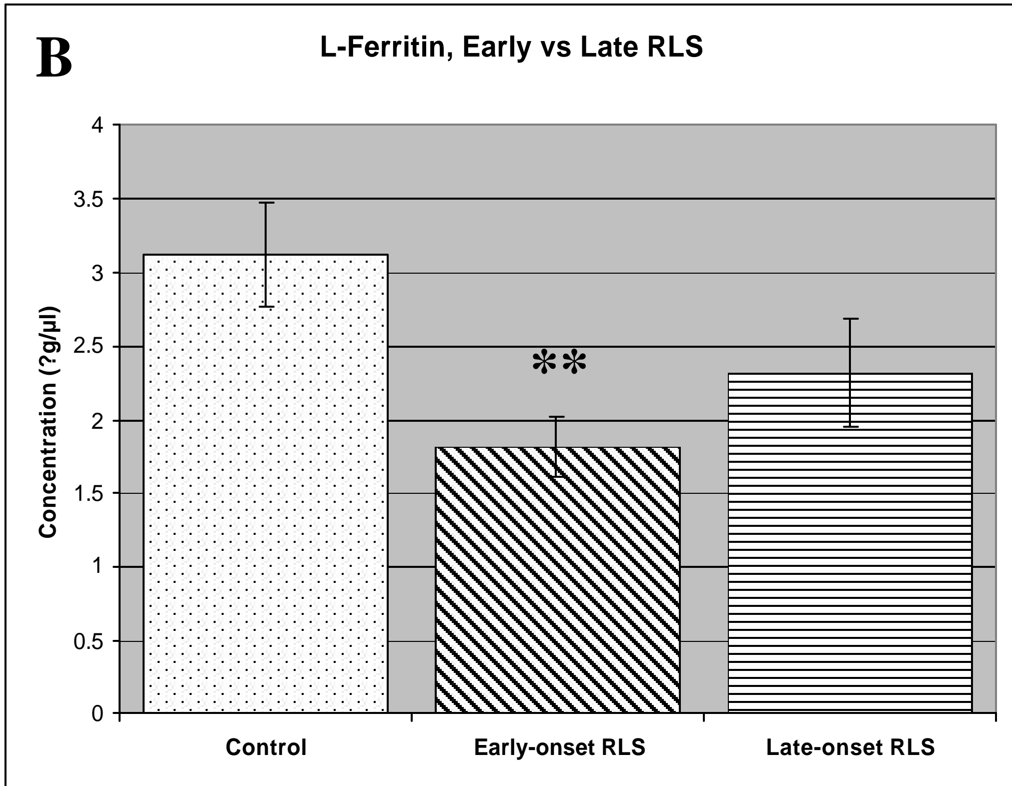


Figure 2.5

Figure 2.6. Protein concentration of CSF samples. The total undiluted CSF protein concentration of each sample is displayed, as quantified by the Pierce Micro BCA Assay. These data demonstrate that there is no significant difference between the mean concentration of total CSF protein in RLS compared to control (718 µg/ml (SEM 30.01) and 659 µg/ml (SEM 83.75), respectively). The differences in the variance between the two groups, however, is significant ($p<0.05$). The SEM is displayed beside each set of data points.

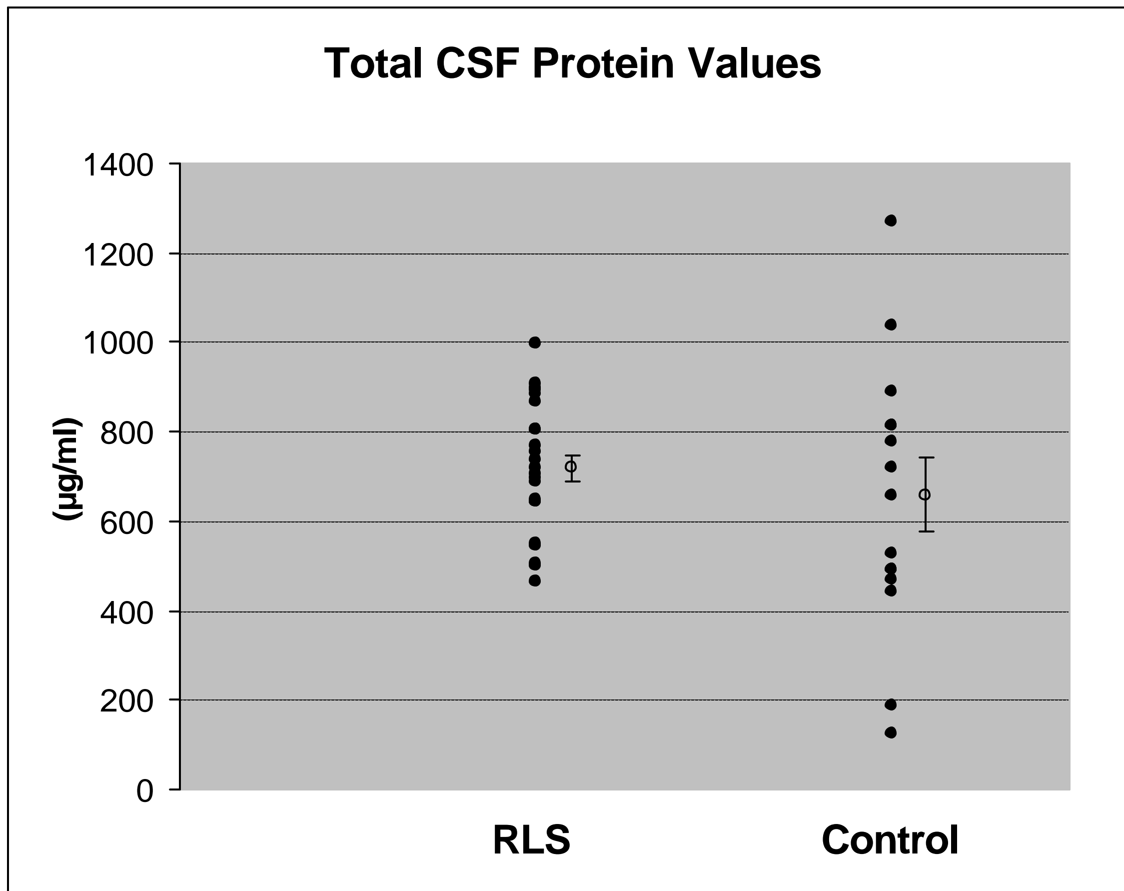


Figure 2.6

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Chapter 3

Hepcidin in the Brain and Cerebrospinal Fluid in Restless Legs Syndrome

Abstract

Restless Legs Syndrome (RLS) is a neurological disorder characterized by a strong urge to move the legs. Sufferers of RLS often experience chronic sleep deprivation, due to the characteristic worsening of symptoms both when at rest and during the night. RLS may be related to iron misregulation in the central nervous system. Evidence from MRI, autopsy, and CSF studies all demonstrate CNS iron deficiency in RLS consistent with a significant abnormality in CNS iron regulation as a putative primary cause of at least some RLS cases. This abnormality occurs consistently in studies of patients with early-onset (before age 45) of RLS symptoms but less so in patients with later onset of symptoms. In this study, we examined the relationship between the iron regulatory hormone hepcidin and primary RLS. Hepcidin is a recently identified iron-regulatory hormone that serves as an intermediary between inflammation, iron regulation, and host defense. To our knowledge, there has been no previous investigation of hepcidin in the human central nervous system. We measured the expression and concentration of hepcidin in the brain and cerebrospinal fluid of both RLS patients and control individuals. In CSF, pro-hepcidin levels were significantly decreased in early-onset RLS patient samples, but not in late-onset RLS patients, when compared to controls. Conversely, in neuromelanin-containing cells, substantia nigra, and putamen, the concentration of hepcidin in samples from RLS patients was significantly increased compared to controls. Our results demonstrate for the first time the presence of hepcidin in the brain, and further, show significant differences in the amount of hepcidin in the brain and pro-hepcidin in the CSF in RLS compared

to control preparations. These data support the mounting evidence that a biological basis for early-onset RLS involves iron mismanagement.

Introduction

The cause of RLS is unknown, but a number of studies using multiple approaches, including autopsy (Connor, Boyer et al. 2003) and MRI (Allen, Barker et al. 2001) have provided evidence that brains of individuals with RLS are iron-deficient. The idea that brain iron is insufficient in RLS is supported by the work in the previous chapter (Clardy, Earley et al. 2005; Earley, Allen et al. 2005), which reports that ferritin (whole molecule), as well as the ferritin H- and L-subunits, in CSF are significantly decreased in RLS, specifically in early- but not late-onset RLS. Work by Mizuno et al (2004) also found a significant decrease in whole molecule ferritin CSF in RLS (Mizuno, Yamashita et al. 2004). The cause of the insufficient brain iron in RLS is under investigation and may involve a defect in an iron regulatory protein (Connor, Wang et al. 2004). This study continues the evaluation of mechanisms involved in regulating brain iron status and their role in RLS.

In this study, we examine the expression and concentration of hepcidin in the brain and cerebrospinal fluid of individuals with primary RLS. Patients with RLS due to, or in conjunction with, other conditions including anemia, renal dialysis, peripheral neuropathy, and neurodegenerative disorder were excluded due to potentially misleading or confounding iron abnormalities. Hepcidin, a recently discovered hormone, may be involved in regulating iron levels in the body, but has not, to our knowledge, been studied in brain tissue. Hepcidin appears to serve as an intermediary between inflammation, iron, and host defense. Hepcidin is an antimicrobial iron-regulatory hormone produced by the liver in response to inflammation. It

inhibits iron transport into plasma from macrophages, hepatocytes, and enterocytes, decreasing the amount of iron available for use in hemoglobin and erythrocytes (Pietrangelo 2004). The decrease in the release of iron into the plasma results in hypoferremia, and if it occurs chronically, manifests as a microcytic anemia that is refractory to iron supplementation, known as the anemia of chronic disease (also called the anemia of inflammation) (Dallalio, Fleury et al. 2003). Gene deletion of hepcidin results in iron loading of the liver (Nicolas 2001). Conversely, transgenic mice that overproduce hepcidin-I (mouse hepcidin) suffer from lethal anemia, and human patients with tumors over-expressing hepcidin exhibit severe anemia (Nicolas 2002; Weinstein, Roy et al. 2002). It is not surprising that erythropoiesis is altered, given that a significant amount of iron is used in hemoglobin synthesis.

The relationship between hepcidin and inflammation has been shown directly by studies demonstrating that interleukin-6 (IL-6) appears to stimulate the hepcidin response in inflammation (Ganz 2005). Experimentally, IL-6 induces hepcidin synthesis in hepatocytes during inflammation; accordingly, if IL-6 action is blocked by antibodies in primary human hepatocytes, hepcidin induction is also blocked. Experiments in humans found that urinary hepcidin levels increased 7-fold after IL-6 infusion, and this increase was accompanied by a 30% decrease in serum iron and transferrin saturation (Nemeth, Rivera et al. 2004).

It is becoming increasingly clear that hepcidin is at the intersection of several pathways involved in the regulation of iron in various organs. Viatte et al (2005) analyzed the level of iron-related proteins in hepcidin-deficient mice and found that the mice expressed increased levels of duodenal Dcytb, divalent metal transporter-1 (DMT1), and ferroportin compared to control mice. Additionally, ferroportin was up-regulated in the spleen and the liver, while ceruloplasmin was decreased in the liver (Viatte, Lesbordes-Brion et al. 2005). Ferroportin (FP),

also known as IREG1/MTP1/SLC11A3, is the first transport protein identified in vertebrates that functions specifically to remove iron from cells (Abboud and Haile 2000; Roy and Andrews 2001).

Iron efflux from cells requires the expression of the cytoplasmic membrane-located exporter protein ferroportin, as well as a ferroxidase — either ceruloplasmin (in macrophages) or hephaestin (in enterocytes) — to deliver ferric iron to transferrin. Hepcidin interacts with ferroportin on the surface of cells. When iron levels in the body are elevated, hepcidin complexes with FP on duodenal epithelial cells and is internalized and degraded, resulting in decreased cellular iron export (Courselaud, Pigeon et al. 2002; Nemeth, Rivera et al. 2004; Knutson, Oukka et al. 2005). In the reticuloendothelial system, elevated systemic iron leads to the formation of a complex of hepcidin and FP on macrophages; the complex is then internalized and iron is sequestered in these cells (Zoller, Theurl et al. 2002; Knutson, Oukka et al. 2005). The result of the FP internalization in both macrophages and duodenal epithelial cells is a decrease in available iron in the bloodstream. This negative feedback system is thought to be a primary mechanism in iron homeostasis (Ganz 2005).

Missense mutations in the FP gene result in autosomal dominant hemochromatosis type 4 (HFE4), which is specifically characterized by reticuloendothelial cell iron accumulation (Pietrangelo 2004). Roy et al (2001) found that HFE4 is caused by defective iron recycling due to the loss of functionality of ferroportin, and that the loss-of-function mutations in the ferroportin gene impair reticuloendothelial iron release (Roy and Andrews 2001; Galli, Bergamaschi et al. 2004).

The current set of studies was designed to determine if hepcidin is expressed in key regions of the brain and cerebrospinal fluid, and to ascertain if significant differences existed

between hepcidin levels in RLS patients and control subjects. Because hepcidin and FP interact, we also evaluated the cellular distribution of FP in the brain. Previously, we published a quantitative analysis of FP in the brain (Connor, Wang et al. 2004).

Materials and Methods

Tissue Sample Analysis:

Sample Procurement

Putamen and midbrain, along with other representative regions of the brain, were obtained at autopsy from the brains of eight patients who had primary RLS meeting the diagnostic criteria established by the NIH workshop on RLS diagnosis (Allen, Picchietti et al. 2003). A detailed report of the neuropathological evaluation of these brains appears elsewhere (Connor, Boyer et al. 2003). The age range of the individuals at death was 53-84 years and all subjects were women with the early-onset phenotype of RLS (onset before age 45 years) (Allen and Earley 2000). The control group consisted of the midbrain from 5 individuals (3 men, 2 women), and the putamen from 8 individuals (4 men, 4 women) who lacked any neurologic history at death and ranged in age from 48-84. The average postmortem interval was 15 h in the RLS group (4–20 h), and 9 h in the control group (4–12 h).

Immunoblot Technique

For the quantitative analysis, substantia nigra and locus ceruleus were dissected from midbrain and putamen sections, respectively, and homogenized, with total protein determination by Bradford assay. A total of 1 μg protein was loaded (500 μl aliquots) in triplicate into a Minifold II slot-blot (immunoblot) system as published previously (Clardy 2005).

Laser Capture Microdissection (LCM) and Protein Isolation

LCM was performed on the substantia nigra from four RLS subjects and four control individuals, as described previously (Connor, Boyer et al. 2003). Briefly, frozen human substantia nigra tissue sections were cut at 20 µm onto LCM slides using a Leica Cryocut 1800 microtome. Neuromelanin-containing cells were captured on thermoplastic polymer-coated caps using a laser beam (7.5 µm in diameter, with 50-85 mW power and 300 ms pulse duration). Approximately one hundred to five hundred neurons per cap were collected from the substantia nigra. Between 1000 and 5000 cells were collected from each substantia nigra.

Neuromelanin-containing cells were resuspended in 50 µl of protein extraction buffer and vortexed. The neuromelanin-containing cells were incubated in PicoPure Protein Extraction Buffer (Arcturus) for 3 hrs at 65 ºC for protein isolation. The samples were vortexed, centrifuged at 2500 g for 2 min and the supernatant was collected. Protein concentration was determined by Bradford assay, and 0.2 µg of total protein were loaded (500 µl aliquots) in triplicate into a Minifold II slot-blot (immunoblot) system as described previously (Clardy 2005).

Immunoblot Analysis

The nitrocellulose membrane was blocked with 5% Blotto, rinsed in PBS/Tween-20 and incubated overnight at 4°C with hepcidin rabbit polyclonal primary antibody (ADI, 1:500). Following PBS/Tween-20 rinses, the membrane was incubated with an HRP-conjugated anti-rabbit secondary antibody (Sigma, dilution 1:5000 in PBS). The membrane was rinsed (PBS/Tween-20), and visualized using an enhanced chemiluminescence kit (KDL). To determine

relative concentrations of standards and samples, the blots were digitized into film images using a Molecular Dynamics Laser Densitometer Model 100A (Sunnyvale, CA) and the densitometric images were then analyzed using PDI Quantity One software (Biorad).

Immunohistochemical Staining

Immunohistochemistry was performed on 15 µm sections from formalin fixed and paraffin samples of substantia nigra. Antigen retrieval was carried out by microwaving slides in 10 mM citrate buffer at pH 6 for 10 minutes. Nonspecific binding was blocked by incubating the sections in a 10:1 solution of methanol and hydrogen peroxide followed by incubation in 2% PBS-milk for an hour. They were then incubated overnight with an antibody to hepcidin (ADI, diluted 1:200) or ferroportin (a gift from Dr. Haile). Sections were then washed in three changes of PBS and incubated for 1 h at room temperature in a 1:200 dilution of biotinylated goat anti-rabbit IgG secondary antibody (Vectastain Elite ABC Kit Rabbit IgG, Burlingame, CA) followed by three PBS rinses to remove unbound secondary antibody. The sections were then incubated for 1 h at room temperature with avidin–biotinylated horseradish peroxidase complex (Vectastain Elite ABC Kit ABC complex, Burlingame, CA). After binding of the complex to the secondary antibody, the reaction was visualized by treatment for 10 min in 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB) solution containing 0.05% hydrogen peroxide. Sections were counterstained with either methyl green or hematoxylin and then coverslipped. Negative antibody control sections were incubated with PBS or pre-immune rabbit serum instead of primary antibody; these showed absence of immunostaining.

Cerebrospinal Fluid Studies:

Sample Procurement

CSF was collected from a total of 46 individuals, 13 with early-onset RLS, 14 with late-onset RLS, and 19 control subjects who did not have RLS. The lumbar punctures (LP) were performed at 10 PM in the evening under an IRB-approved protocol. The patients met the standard characteristics described previously (Clardy 2005; Earley 2005). Briefly, RLS subjects met all criteria for the syndrome (Connor, Boyer et al. 2003), reported nightly symptoms prior to treatment for at least six months, showed a positive clinical response to dopaminergic agents, and had periodic limb movements during sleep (PLMS) >20/hr on an all night polysomnogram when off of medications. Patients who developed RLS secondary to other specific causes or who had other obvious medical problems complicating evaluation of RLS symptoms, iron status, or sleep were excluded from the study. Control subjects had no symptoms to suggest RLS; no first degree relatives with RLS; and PLMS <15/hr (on screening or on PSG). Subjects were matched for age, race, and gender. Exclusion criteria included the presence of sleep apnea/hypopnea rates of >30/hr, serum ferritin <18 µg/l; percent hemoglobin iron saturation <16%. RLS and psychoactive medications (except nicotine and caffeine) as well as iron-containing supplements were stopped 2 weeks or 6-half-lifes (which ever was the longest) prior to admission to the GCRC for the study. The patient's age of onset of RLS symptoms was obtained in the clinical interview conducted by an expert in RLS board-certified in sleep medicine at the time of the patient's admission to the GCRC prior to obtaining any of the data used in this study. The early-onset RLS group includes patients with symptom onset before the age of 45; patients with symptom onset after age 45 are included in the late-onset RLS group. This is an IRB-approved study, with research carried out according to the principles of the Declaration of Helsinki.

Informed consent was obtained from all participants, and appropriate consent was obtained for the use of all tissue.

ELISA Analysis

We utilized the DRG Hepcidin Prohormone ELISA (EIA-4015, DRG International, Inc.) for the detection and determination of pro-hepcidin in the CSF of RLS patients and controls. The wells are coated with a polyclonal rabbit antibody directed against hepcidin-(28-47) antibody. Procedures were followed as outlined in the manufacturer's protocol. All steps were performed at room temperature. Briefly, assay buffer, pro-hepcidin standards and controls, undiluted CSF samples, and biotin conjugate were added into appropriate wells. The plate was mixed for ten seconds and incubated at room temperature for one hour. After the one hour incubation the microtiter plate was washed. Next, the wells were rinsed three times with wash solution, 400 µl per well each rinse. After rinsing, 100 µl of the streptavidin HRP complex was added to all wells, incubated for 30 minutes at room temperature, rinsed, and followed by the addition of 100 µl of substrate solution to each well. After ten minute incubation, 100 µl of stop solution was added to each well and the absorbance was determined at 450±10 nm.

Statistics:

Immunoblot results were calculated as the average optical density (OD) for the triplicate aliquots for each sample was calculated. Results are presented as relative values with respect to control patients (RLS vs. Control). Statistical analyses used the Student's t-test.

The CSF concentration of pro-hepcidin as determined by ELISA is inversely proportional to the optical density measured. Thus, the standard curve was constructed using a four parameter logistic function, with the average absorbance (Y) of each reference standard plotted against its corresponding concentration (X) in ng/ml. For CSF analyses prior studies showed clear iron deficiencies for early-onset RLS compared to controls and therefore we hypothesized that early-onset RLS compared to controls would have reduced pro-hepcidin. The pro-hepcidin values were skewed and not normally distributed, we therefore tested our primary hypothesis using the non-parametric Mann-Whitney U test. We similarly evaluated as an exploratory analysis possible significant differences between late-onset RLS and controls

Results

Immunoblot analysis identified a significantly increased concentration of hepcidin in RLS samples compared to controls in both neuromelanin-containing cells in the substantia nigra isolated by laser capture dissection (Figure 3.1A, $p < 0.001$) and in homogenates obtained from the substantia nigra (Figure 3.1B, $p<0.05$) and locus ceruleus (Figure 3.1C, $p<0.01$).

On microscopic assessment of the immunostained brain slides, the neurons in the substantia nigra expressed hepcidin in both the control and RLS tissue, and the distribution pattern within the neurons was similar (Fig. 3.2A and 3.2B). The staining was relatively light and confined to the soma in the control substantia nigra. The immunoreaction product for hepcidin was routinely more intense in the RLS tissue compared to control, and extended into the primary processes of neurons.

We utilized a pro-hepcidin ELISA assay to analyze the cerebrospinal fluid of early-onset RLS, late-onset RLS, and control samples. As shown (Table 3.1), CSF pro-hepcidin levels were

significantly decreased compared to controls in early-onset RLS patient samples (p=0.04), but not in late-onset RLS patients (p=0.31).

Hepcidin is thought to interact with ferroportin (FP) to regulate iron efflux from cells. Because we detected pro-hepcidin in the CSF, we performed an immunocytochemical analysis and demonstrated that FP was expressed in the choroid plexus and ependyma (Figure 3.3).

Discussion

These results demonstrate that hepcidin is increased in the brain parenchyma and within the neuromelanin cells of the substantia nigra of individuals with RLS. In contrast, within the CSF, the pro-hepcidin levels are decreased in RLS, but only in individuals with early-onset RLS. In individuals with last onset RLS, the hepcidin levels in the CSF do not differ from controls. In the autopsy study, all of the analyses were performed on individuals with early onset RLS. Because of the suspected role of hepcidin in iron regulation, these data provide compelling support for the idea that iron status in the brain is altered in individuals with RLS, specifically early-onset RLS.

This is the first study to our knowledge to examine hepcidin in the brain and in a neurological disorder. The source of hepcidin in the brain and CSF is not known. Therefore, although the data support the evolving concept of altered brain iron status in RLS, it is a challenge to interpret whether the different directions in which hepcidin changed in RLS in the CSF and brain parenchyma indicate a problem with this iron regulatory mechanism or an adaptive response to the altered iron status. To interpret the meaning of the hepcidin results, the existing mechanistic knowledge regarding hepcidin and iron at the level of the gut provides clues to a potential function of hepcidin in the brain. Nemeth et al reported that in enterocytes,

hepcidin binds directly to ferroportin, causing ferroportin to be internalized and degraded (Nemeth, Tuttle et al. 2004). This ferroportin internalization effectively halts cellular iron export. For enterocytes, this mechanism can serve as a way to prevent iron overload, because the enterocytes with internalized ferroportin are shed about every two days; the internalized iron is thus shed as part of the enterocyte package. Therefore in order to attempt to interpret the hepcidin results we included the analysis of ferroportin in the brain.

Ferroportin was found in the choroid plexus, ependymal cells lining the ventricles, and in neuromelanin-containing cells in the substantia nigra. The detection of ferroportin in the neuromelanin-containing cells agreed with our previously published immunoassay data on laser captured neuromelanin-containing cells (Connor, Wang et al. 2004). The detection of ferroportin in the choroid plexus and ependyma is in agreement with studies on rat brains (Wu, Leenders et al. 2004). There is not agreement in rodent studies on the distribution of ferroportin on brain cells. Burdo et al (2001) reported that ferroportin staining is found in most rat brain regions, and is particularly robust in the pyramidal neurons of the cerebral cortex (Burdo, Menzies et al. 2001) and could not be detected in glial cells in the rat brains even under conditions of low brain iron using Belgrade rats as a model. Wu et al (2004) also characterized the distribution of FP in the rat brain and found the distribution of FP in neurons as reported in Burdo et al (2001), but additionally found FP in glial cells. We did not observe ferroportin in glial cells in the human tissue. The increase in hepcidin in the parenchyma in RLS could serve as a mechanism to maintain iron in the neurons, similar to the mechanism by which hepcidin interacts with FP in the enterocytes. We propose (Figure 3.4) that hepcidin binds to the ferroportin on the cell surface, causing ferroportin internalization and effectively halting iron export. The increased staining intensity of hepcidin in the neuromelanin-containing cells in our study would be

consistent with this interpretation of our data. The most pressing question raised by the data is how hepcidin levels become increased in the brain tissue. Future studies should address this question.

In the CSF, the pro-hepcidin levels are decreased in the individuals with early onset RLS. The decreased level of pro-hepcidin in the cerebrospinal fluid of early-onset RLS patients is consistent with previous findings by our group and others (Mizuno, Yamashita et al. 2004; Clardy 2005; Earley 2005), namely that alterations in iron regulatory mechanisms or iron status can be detected in the CSF in early-onset RLS but not late-onset RLS. There is relatively little understanding about the role of the CSF and the choroid plexus in brain iron homeostasis. The choroid plexus contains transferrin (Tf) mRNA, and Tf is secreted from the choroid plexus (Moos and Morgan 2000). Experiments with hypotransferrinemic mice have demonstrated that iron uptake into the choroid plexus can occur independent of transferrin (Malecki, Cook et al. 1999; Burdo, Antonetti et al. 2003; Deane, Zheng et al. 2004; Beard, Wiesinger et al. 2005). Ferritin is also found in the choroid plexus, suggesting that this organ can store iron and may be a regulatory site in brain iron acquisition, at least for the CSF. We demonstrated that FP is present in the choroid plexus, suggesting that FP could be a mechanism for iron release from these cells. It is not known at this time why iron release from choroid plexus would involve both transferrin secretion and release through FP. Perhaps Tf is released in the "apo" form and complexes with iron extracellularly in the CSF. Ceruloplasmin, an important ferroxidase that promotes iron-Tf interaction, is found in the CSF.

The reduced CSF levels of hepcidin could have the effect at the choroid plexus of increasing iron release, as hepcidin would not be available to bind with ferroportin. Decreased hepcidin in the CSF could be an attempt to correct the iron imbalance in RLS; if the decrease in

hepcidin is successful in supporting an increase in iron release from the choroid plexus to raise the CSF iron content, Tf in the CSF may in turn distribute this to iron-poor regions of the brain. Alternatively, the CSF could be a route of iron efflux from the brain, and thus the CSF-hepcidin signaling could be exacerbating RLS by further depriving the brain of iron.

Similarly, in regard to the transport of iron at the ependymal layer, where we demonstrated the presence of ferroportin, hepcidin would be expected to have an effect on iron movement. There is little knowledge about the movement of iron from the CSF into the brain across the ependymal layers. In studies of developing rat brain, Siddappa et al demonstrated the expression of DMT1, transferrin receptor, and the iron regulatory proteins in ependymal cells, indicating that iron uptake and regulation occurs in these cells (Siddappa, Rao et al. 2003). We have also demonstrated that DMT1 is present in the ependyma cells in the human brain (Connor, Menzies et al. 2001). If rats are made iron-deficient by dietary manipulation, there is no detectable change in the expression of the regulatory proteins or transferrin receptor in ependymal cells by immunostaining, suggesting that systemic iron changes do not impact CSF iron transport or efflux through the ependymal layer (Siddappa, Rao et al. 2003). After intracerebroventricular injection of [59Fe125I]transferrin, there was a higher accumulation of 59Fe than of [125I]transferrin in the brain, but both iron and transferrin were found only in areas close to the ventricles and pia, suggesting that the movement of iron into the brain was no greater than expected for diffusion (Moos 2002). This latter study led to the interpretation that transferrin is not a significant means of iron transport through the ependyma for the brain, but rather that the likely primary role of CSF transferrin is to neutralize and export iron to the blood (Moos 2002). Hepcidin could serve to limit iron release from the brain into the CSF or could serve to hold iron in the ependymal cells. Given the existing lack of data on iron movement into

or out of the CSF, it is not possible to interpret the hepcidin changes in the CSF as part of a supportive adaptive response to low brain iron or as a contributor (or even cause) of the low brain iron in the RLS brain. The results we report herein provide a compelling argument for the need for more research into the role of CSF in maintaining brain iron homeostasis.

The assay used in the CSF study was an ELISA that targets pro-hepcidin, whereas the brain parenchymal levels were determined with an antibody to hepcidin. This latter antibody was used to maintain consistency with the antibody used for immunohistochemistry. The utility of comparing hepcidin and pro-hepcidin levels has been questioned (Brookes, Sharma et al. 2005). Brookes et al point out that the pro-hepcidin assay contains the N-terminus, and may not detect the C-terminus of mature hepcidin. Utilizing the pro-hepcidin assay, however, Kulaksiz et al report findings that agree with work done with the processed hepcidin hormone, including decreased pro-hepcidin levels in HH (Kulaksiz, Gehrke et al. 2004). We are unaware of any evidence suggesting that pro-hepcidin levels are not reflective of hepcidin measures.

In conclusion, we provide evidence for hormonal iron signaling in the CNS by demonstrating the presence of hepcidin in the brain and significant differences in the amount of hepcidin in the brain and pro-hepcidin in the CSF in RLS compared to controls. These data clearly showed hepcidin is responsive to the iron-related changes in RLS, but it remains to be determined if hepcidin secretion is pathological or compensatory in RLS. Future investigation of the source of hepcidin in brain tissue as well as understanding its role in brain iron regulation is warranted.

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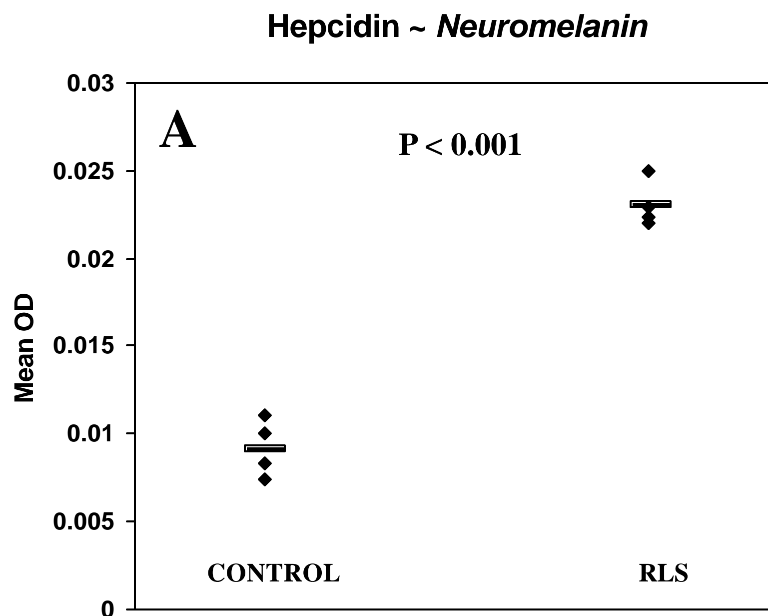
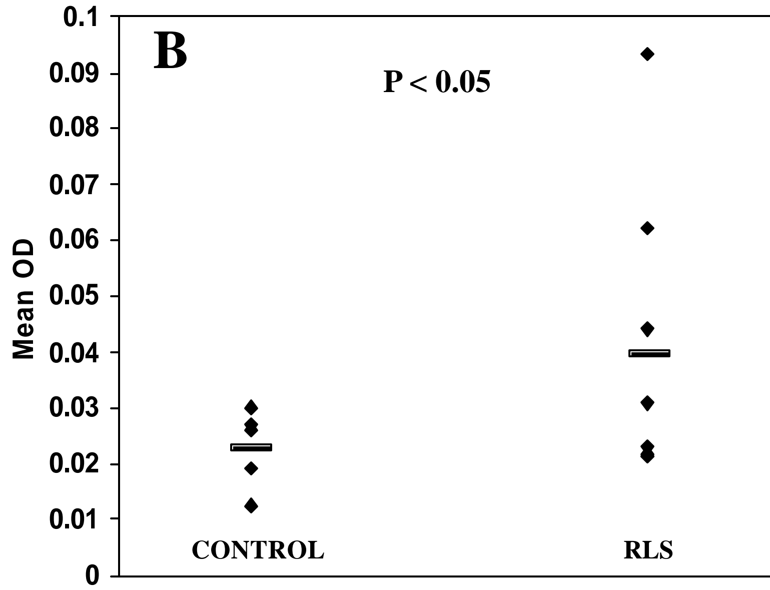
Figure 3.1. Immunoblot for hepcidin in neuromelanin cells, substantia nigra, and putamen from the brain of RLS patients compared to controls. Immunoblot analysis measuring hepcidin levels in neuromelanin-containing cells from the substantia nigra isolated by laser capture microdissection (A), and in dissected regions of substantia nigra (B) and putamen (C) from brains of RLS patients compared to controls. In all three brain regions, RLS patients have significantly lower levels of hepcidin than controls ($p<0.001$, $p<0.05$, and $p<0.01$, respectively).



Hepcidin ~ *Substantia Nigra*



Hepcidin ~ *Putamen*

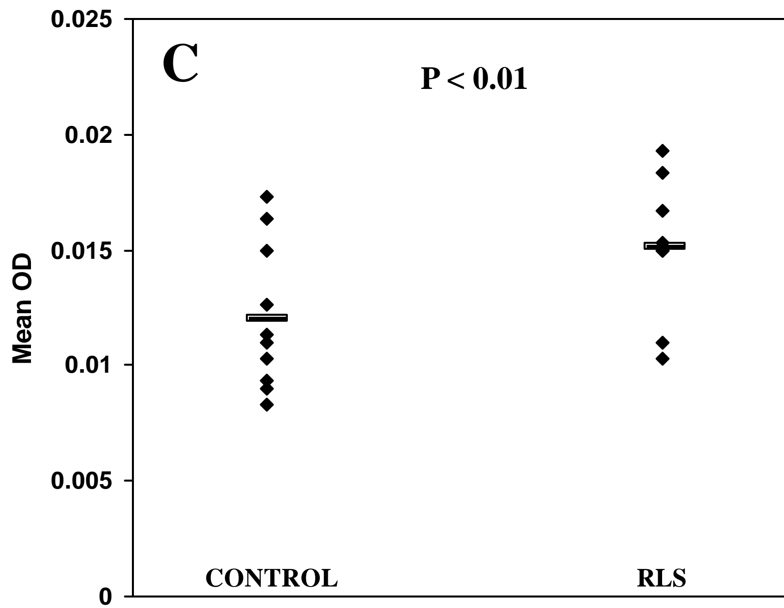
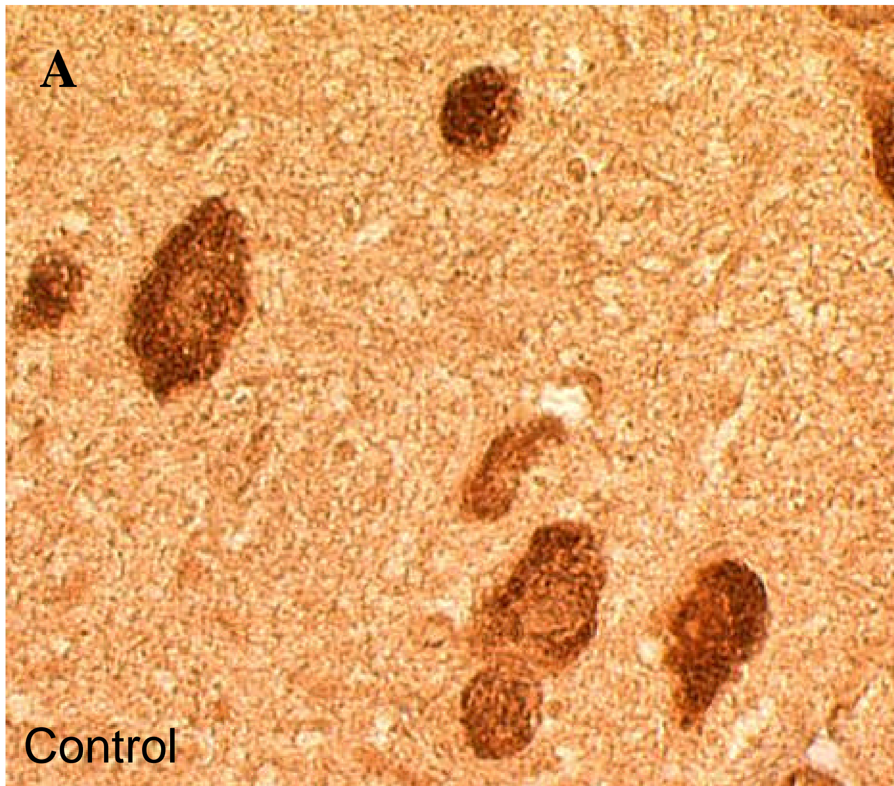


Figure 3.1

Figure 3.2. Micrographs of cellular distribution of hepcidin in the neurons of the substantia nigra of control and RLS patients. Cellular distribution of hepcidin in the neurons of the substantia nigra of control (Panel A) and RLS patients (Panel B). The immunoreaction product is primarily found in the neuromelanin containing cells in both control and RLS brains. The immunoreaction product for Hepcidin is visible in the soma and primary dendrites (arrows) of the neurons and is more robust in the neurons in the RLS which is consistent with the quantitative findings. The Hepcidin positive processes in the neuropil are also much more striking in the RLS brain than in the control brain. Scale bar: 8 μm.



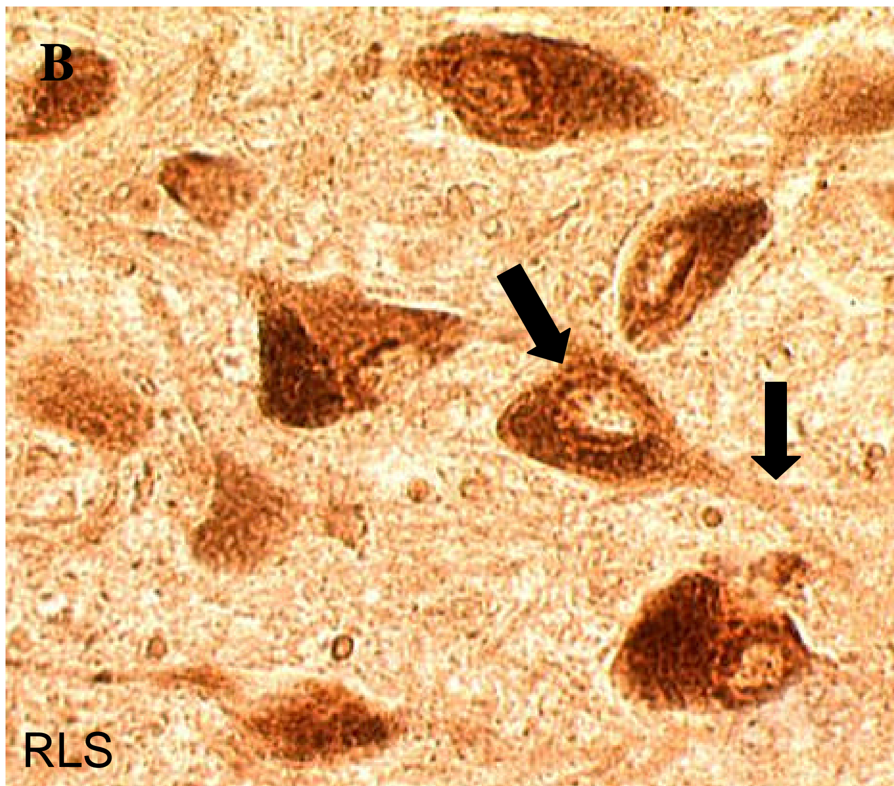


Figure 3.2

Figure 3.3. Micrograph of the distribution of ferroportin (FP) human choroid plexus and ependymal cells. The distribution of ferroportin is shown in the human choroid plexus (A) and ependymal cells (B). The epithelial cells of the choroid plexus are positive for FP in this paraffin embedded section from human brain. The immunoreaction product is orange-brown. The tissue has been counterstained with hematoxylin so that the nuclei (blue) are visible. (B) Ependymal cells lining the ventricles stain for FP. The immunoreaction product is red and the tissue has been counterstained with hematoxylin so that the nuclei are visible (blue).

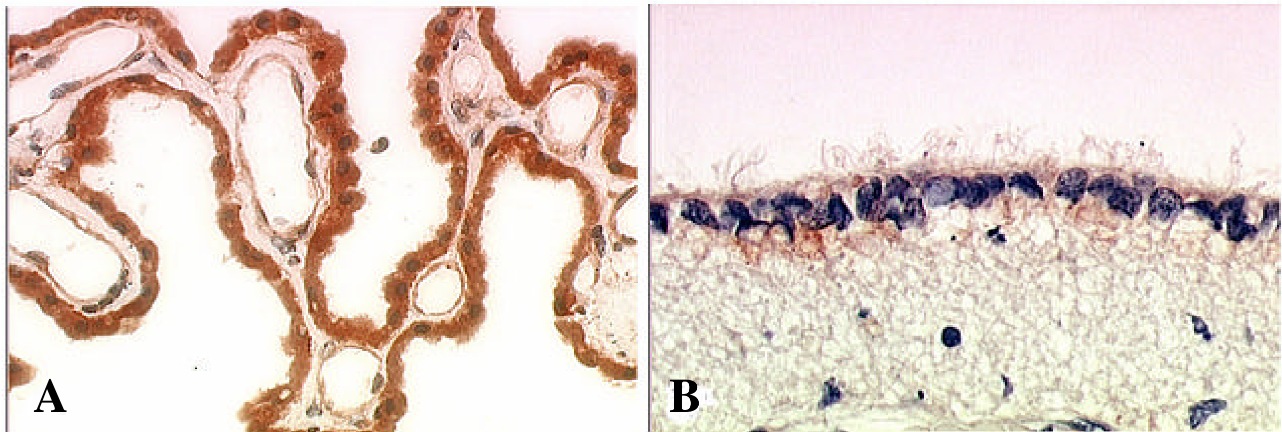
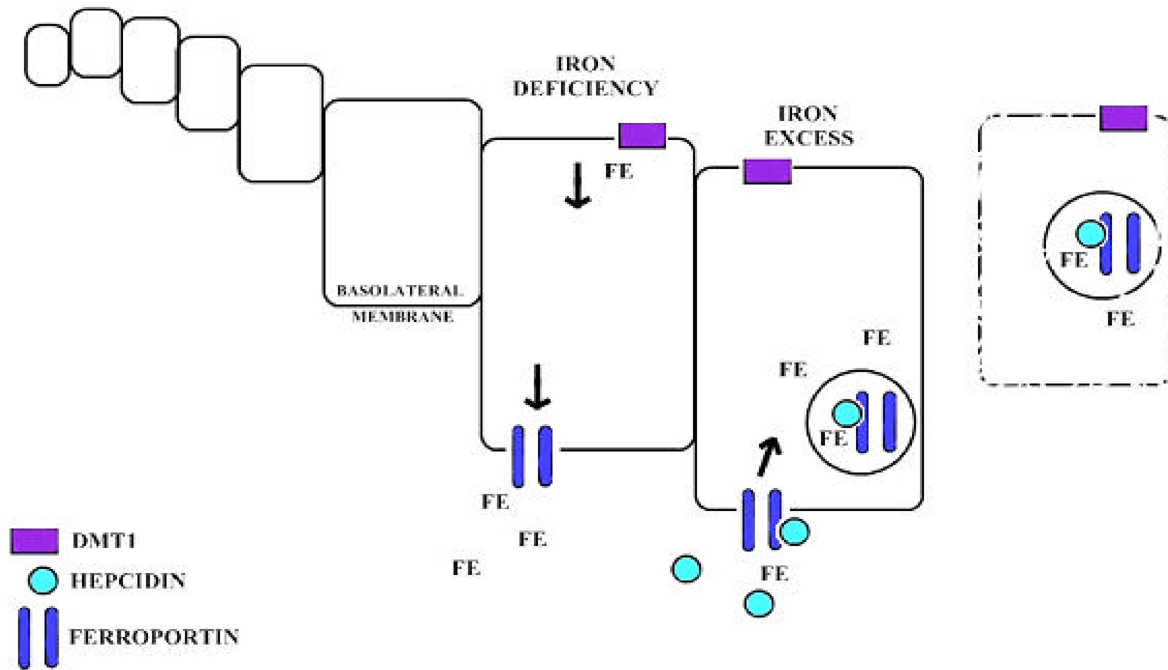


Figure 3.3

Figure 3.4. Schematic of the proposed mechanism of action of hepcidin in both the small intestine and the brain. The proposed mechanism of hepcidin action in both the small intestine and the brain are based on the model of Ganz (Ganz, 2005). In the enterocytes of the small intestine, hepcidin binds ferroportin, causing ferroportin internalization and effectively halting iron export. For enterocytes, this mechanism can serve as a way to prevent iron overload, as the enterocytes with internalized ferroportin are shed about every two days; the internalized iron is thus shed as part of the enterocyte package. In the brain, formation of the hepcidin-FP complex in brain also causes ferroportin internalization into cells (neuromelanin-containing cells, for example). Given limited cell turnover, the same mechanism could have the opposite effect, resulting in the retention of iron instead of iron loss.

INTESTINE



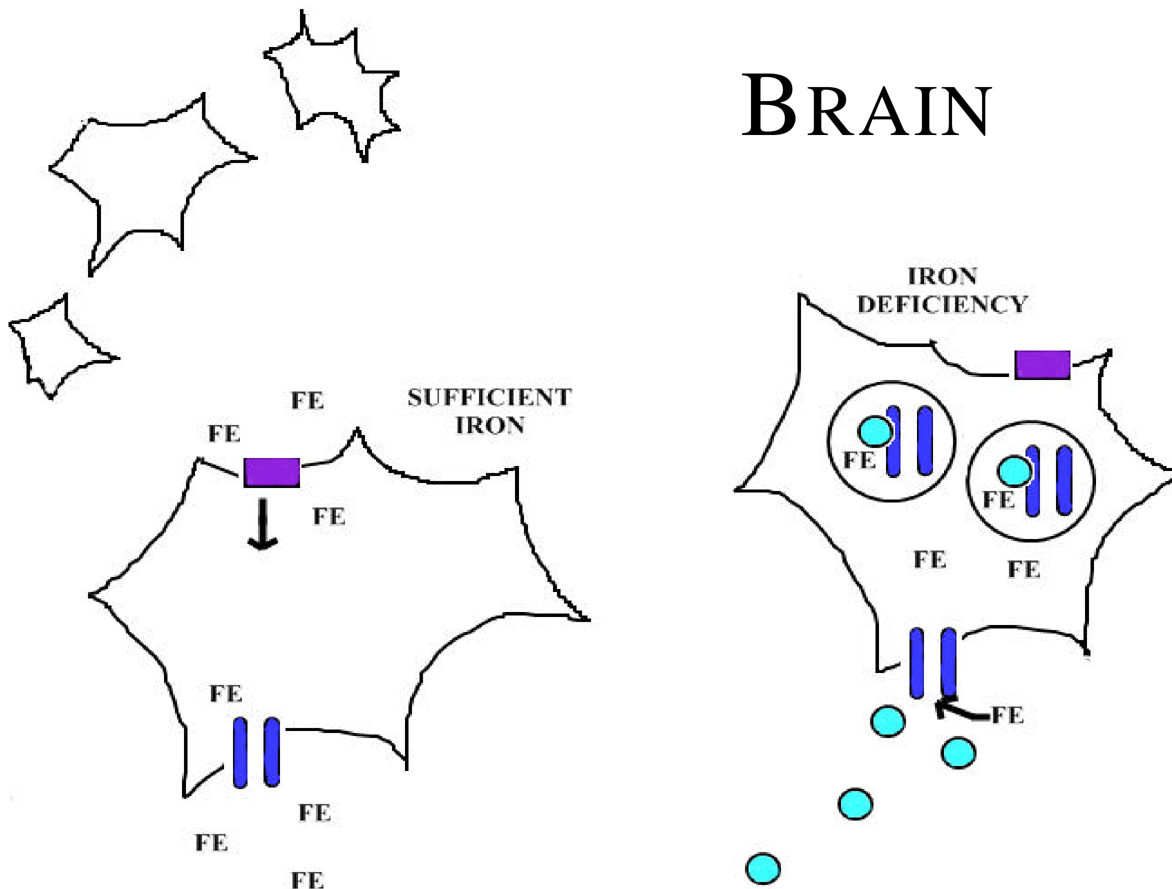


Figure 3.4

Table 3.1. CSF Pro-hepcidin mean and standard error of the mean for each diagnostic group.

	Number in Group	Mean	SEM	U, p*
Controls	19	49.9	13.2	----
Early-onset RLS	13	22.0	8.2	71, p=0.044
Late-onset RLS	14	29.8	7.4	105, p=0.31

* Mann-Whitney U comparison with controls.

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Chapter 4

Acute and Chronic Effects of Developmental Iron Deficiency on mRNA Expression Patterns in the Brain

Abstract

Because of the multiple biochemical pathways that require iron, iron deficiency can impact brain metabolism in many ways. Most studies on iron deficiency in the brain have focused on neurotransmitters and myelin. To obtain a broader spectrum of the effects of iron deficiency and to expand the existing studies to mRNA expression patterns we used gene array expression analysis. The goal of this study was to identify a molecular footprint associated with the ongoing versus long term consequences of iron deficiency. Rats were born to iron-deficient mothers, and were analyzed at two different ages: at 21 days, while weaning and iron-deficient; and at six months, after a recovery period consisting of normal iron intake for five months. Approximately 300 genes were significantly changed in the 21-day animals. Several gene clusters were identified, including: myelin-related, signal transduction, channel/pore class transporter and alpha-type channel activity, ion channel activity, DNA binding, transitional metal binding, and solute carrier family members. In the six month animals, only twelve genes were identified as significantly changed, but reflected changes that are long-term. Of the twelve genes found, all were down-regulated, and seven were expressed sequence tags or transcribed sequences, leaving five genes whose function can be discussed. Of the protein products of these five genes, two are cytoplasmic, two are nuclear, and one is found in both the nucleus and cytoplasm. None of these five genes were changed in the 21-day animals. The down-regulation of these five genes from the six month animals represent possible compromises in cytoskeletal stability and neuronal infrastructure, decreased nucleic acid translation, and less protein

degradation. Overall, the data indicate that early iron deficiency impacts many genes, most of which are not directly iron-related, whereas the long term consequences of iron deficiency on gene expression are more limited. These data suggest that the gene array profiles obtained at postnatal day 21 reflect a brain under development in a metabolically compromised setting that given appropriate intervention is mostly correctable. There are, however, long term consequences to the developmental iron deficiency that could underlie the neurological deficits reported for iron deficiency.

Introduction

Insufficient amounts of body iron affect up to one billion people worldwide (Andrews 2000). Iron is especially crucial during development, as it is required for proper myelination, and is also a cofactor for enzymes in neurotransmitter synthesis (Larkin 1990; Beard and Connor 2003; Beard, Wiesinger et al. 2003). Studies in animals dating back over three decades have documented the damage resulting from iron-deficiency. Dallman et al demonstrated that even after iron-deficient rats were returned to a normal iron diet for several days, non-heme iron remained depressed, as did ferritin in brain (Dallman, Siimes et al. 1975). Weinberg et al also noted that rats exposed to iron deficiency for the first 28 days of life never recovered, exhibiting a persistent deficit in brain non-heme iron, as well as changes in behavior and physiological responsiveness (Weinberg, Levine et al. 1979). Further behavioral effects, specifically in the dopaminergic system, were found in another study of rats that were iron-deprived for 28 days postnatally. The rats exhibited decreased motor activity and reversed circadian rhythms of thermoregulation and motor activity, indicating the major role iron plays in the normal function of the dopaminergic system in the brain (Youdim, Yehuda et al. 1981). More recent animal studies have distinguished differences between pre- and post-natal recovery from iron deficiency.

If the iron deficiency occurs during neonatal and post-weaning periods, recovery upon iron repletion (with the iron sufficient diet given two-to four weeks postnatally) occurs quickly (Chen, Connor et al. 1995; Erikson, Pinero et al. 1997; Pinero, Hu et al. 2000), but the effects of iron deficiency *in utero* appear irreversible, even after iron repletion (Felt and Lozoff 1996; Kwik-Uribe, Gietzen et al. 2000; Kwik-Uribe, Golub et al. 2000).

In humans, a study of second grade children who were anemic in infancy revealed that the learning achievement score and the positive task orientation was significantly lower in the anemic group than in the non-anemic control group. Data were controlled for maternal education and sex of child (Palti, Meijer et al. 1985). A more recent study by Lozoff et al found that children who had iron deficiency in infancy scored lower on tests of mental and motor functioning as teens (greater than ten years later) than those infants who were not iron-deficient as infants. Additionally, more of the previously iron-deficient infants had repeated a grade, been referred for special services, and/or was the object of parental and teacher concern regarding anxiety/depression, social difficulties, and attention problems (Lozoff, Jimenez et al. 2000). A study of iron-deficiency anemia in young South African anemic mothers of full-term infants found that the infants of anemic mothers were developmentally delayed at 10 weeks. At 9 months, despite normalization of iron status in some mothers, the developmental delays were not diminished in the infants. The iron deficiency also affected the mothers, and even 9 months post-partum, anemic mothers were more negative, less engaged, and less responsive toward their babies than were control mothers. Mothers treated for iron-deficiency exhibited behaviors similar to controls (Perez, Hendricks et al. 2005). A study of South African women by Beard and colleagues (2005) found that iron treatment of anemic mothers up to 9 months postpartum resulted in 25% improvement of depression, stress, and cognitive function, further suggesting the

importance of maternal iron status on infant development (Beard, Hendricks et al. 2005). These studies highlight the need to study iron-deficiency over time, from infancy onward, and hint at the differences between ID at different stages in life (birth, infancy, and motherhood).

It is now accepted that dietary iron-deficiency lowers brain iron and interferes with protein synthesis in the brain (Beard and Connor 2003). There is, however, a notable lack of research into the effects of such early iron deficiency in later, adult life. In these studies, we obtained a gene expression profile of rat brain to identify the molecular footprint of the iron-deficient brain. This information may point to the specific pathways causing the underlying pathophysiology of both acute iron deficiency and its long-term, chronic effects. A benefit of microarrays in the analysis of iron-deficient brain is ability to investigate global gene changes without the bias of *a priori* hypotheses. We also wanted to examine as broad a range of systems as possible in this first pass analysis in the iron-deficient brains, and thus we tested the entire brain in our analysis to gain an understanding of the most dramatic and global changes caused by iron deficiency.

Methods and Materials

Animal Characteristics - The studies reported here were performed in compliance with the animal procedures approved by the University of Michigan institutional animal use committee (protocols 2002-129 and 7623). Pregnant Sprague-Dawley dams were fed an iron-deficient diet (4-10 mg/kg iron) or an iron-sufficient diet (40 mg/kg iron) beginning at gestational day five. Diets were prepared by Harlan Teklad Nutritionals (Madison, WI). Mothers and litters were maintained on their diets through gestation and lactation. After postnatal day 20, all animals were fed the iron-sufficient diet. The first experimental group was sacrificed at 21-days of age.

For the second group of animals, pups were weaned at postnatal day 23 and maintained on the iron-sufficient diet until they were killed at six months of age. There were 4 animals per group for the 21-day analyses and 4 animals per group for the 6-month analyses.

RNA Preparation and Microarray Analysis - Frozen brain tissue was homogenized and extracted with TRIzol reagent (Gibco BRL Life Technologies, Baltimore, MD, USA) and further purified with the Qiagen RNeasy kit (Qiagen, Valencia, CA) utilizing the clean-up step according to the manufacturer's instructions. RNA quality was evaluated by A260/A280 ratio. Randomly selected samples were further analyzed for RNA integrity using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA); all samples analyzed demonstrated acceptable quality. Synthesis of double-stranded DNA and biotinylated cRNA from total RNA, and its subsequent hybridization to the chips, were performed according to Affymetrix (Santa Clara, CA) recommendations. Streptavidin-phycoerythrin staining of arrays after hybridization also followed Affymetrix recommendations. Biotin-labeled and fragmented RNA was hybridized to Rat Genome U34A Gene Chips containing approximately 7000 full-length sequences and 1000 expressed sequence tag (EST) clusters. The microarrays were scanned by the Affymetrix gene array system (http://www.affymetrix.com). Preliminary data analyses were performed with the Affymetrix GCOS software package and the Affymetrix Data Mining Tool.

Real-time PCR - Total RNA was prepared for qualitative real-time PCR using Invitrogen's SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen Corporation, Carlsbad, CA). PCR was performed using TaqMan TAMRA detection probe and primers either purchased from inventory or designed with the online software Custom TaqMan® Gene Expression Assay

File Builder (Applied Biosystems, Foster City, CA). Real-time PCR amplification of cDNA was performed using TaqMan Universal PCR Master Mix (Applied Biosystems). Values were normalized to those obtained for ribosomal 18s mRNA. For further information on the rationale for selection of the TaqMan system, please see the Appendix, section two.

Statistical Analysis - Before testing significant changes in gene expression, expression signals were normalized by using the R-Affy package from Bioconductor (version 1.1, Irizarry et al. 2003) to remove background noise and non-biological variations among arrays. The background noise was removed from the PM (perfect match) probe intensities using the "RMA" method (Irizarry), which assumes a global model for the distribution of probe intensities and models the PM probe intensities as the sum of a normal noise component and an exponential signal component. Normalization was performed according to the quantile normalization method (Bolstad 2003). Quantile normalization assumes that the expression of majority of genes on the arrays does not change in different treatments, and that the distribution of probe intensities for each array in the dataset is the same. For each probe set, the logs of the background corrected, normalized PM intensities were fitted with an additive multi-chip linear model, which had a chip-wise abundance term and a probe-wise affinity term. The robust median polish procedure was utilized for fitting (Tukey 1977). Estimates were reported on the log2 scale. Because approximately thirty percent of the probes were found with MM (mismatch) > PM, and there existed a high correlation between PM and MM intensities, the use of MM as internal control was questionable. Therefore, expression values were obtained based on PM intensities not PM-MM intensities.

Significant gene expression alterations were identified using Significance Analysis of Microarrays (SAM) computer software (Tusher, Tibshirani et al. 2001). SAM assigns a score to each gene on the basis of gene expression change relative to the standard deviation of repeated measurements and identifies genes with statistically significant changes in expression using a permutation procedure. SAM controls the false positives resulting from multiple comparisons through controlling the false discovery rate (FDR) (Benjamini 1995). FDR is defined as the expected proportion of false positive genes among all genes that are considered significant. The FDR was set at 0.05 for the 21 day animals and 0.063 for the six month animals. Computer software dChip (Li and Wong 2001; Li 2003) version 1.3 was used to cluster samples and genes, and to identify functionally significant gene clusters. Normalized chip intensity data were imported into dChip. Gene information file for Affymetrix mouse genome RG U34A array was obtained from dChip's website at www.dChip.org. Hierarchical clustering was used for gene clustering. After clustering on genes, dChip systematically assesses the significance of all functional categories in all branches of the hierarchical clustering tree. Finite sampling and hypergeometric distribution was used to measure the significance. P-value is the probability of seeing x genes of a certain function occurring in a cluster of k genes at random, given n annotated genes on the array or of a list of genes, of which m genes have a certain function. Clusters with p-values < 0.005 were considered significant and were reported. The less than 0.005 rule was suggested by the dChip program. The p-values were unadjusted for multiple comparisons. Therefore, a more stringent rule (less than 0.005) need to be used instead of the usual less than 0.05 rule.

For statistical analysis of the real time PCR data, the Sequence Detection Software (ver. 1.2.3) provided by Applied Biosystems was used to export the raw data for each 96-well plate.

Each plate had 6 sets of 12 numbers: 1 set for Gene 1 control, 1 set for Gene 1 target (iron-deficiency), 1 set for Gene 2 control, 1 set for Gene 2 target, 1 set for 18S control, and 1 set for 18S target. For each set, an outlier was identified if it was not in the range of mean-2 X standard deviation and mean+2 X standard deviation. Outliers were then omitted manually using the Sequence Detection Software. The RQ value was then recalculated using the software. Results were exported from the software for each of the 12 plates. Most sets did not have any outliers (the use of +/- 3 times standard deviation as the limits did not yield any outliers).

Results

The statistical analysis revealed 334 significantly changed genes in the 21-day rats. These genes are listed in Supplemental Table 4.1. To meet significance, the genes had an FDR of <0.05 (the expected proportion of false positive genes among all genes that are considered significant). The 50 most dramatically changed genes from the 334 significantly changed genes — the 25 most up-regulated and the 25 most down-regulated — are shown in Table 4.1. Several significant gene clusters were identified from the 334 significantly changed genes, including: myelin-related, signal transduction, channel/pore class transporter and alpha-type channel activity, ion channel activity, DNA binding, transitional metal binding, and solute carrier family members (Figure 4.1). Clusters with p-values < 0.005 were considered significant. The identity of the genes in each cluster, the fold change, and the q-value are displayed in Table 4.2.

From the over 300 genes identified in the 21-day animals, twelve genes were selected for verification by real-time PCR. These twelve genes were chosen based on several criteria. The genes tested for verification were not only those genes with the most the dramatic fold change and q values, but also included genes considered to be less significantly changed according to the fold change and q-value. Some of the genes were selected as possible candidates for iron-related

disorders, including possible relationship to iron deficiency based on QTL analyses (for example, see (Jones, Reed et al. 2003)). Of the twelve genes selected for PCR analyses, six were up-regulated on the microarray studies and six were down-regulated. The verification results are shown in Figure 4.2A.

In the six-month-old animals, a total of twelve genes were identified as significantly changed. Of the twelve genes found, all were down-regulated, and seven were expressed sequence tags or transcribed sequences, leaving five genes for further investigation. Of the five genes, two of the protein products are cytoplasmic, two are nuclear, and one is found in both the nucleus and cytoplasm. The results of the real-time PCR verification of all five genes are shown in Figure 4.2B. Real-time PCR was additionally performed on biological replicates of the 21-day-old animals, and the results are displayed in Figure 4.3. Brains from eight different animals (four control, four iron-deficient) were used to verify the changes in each gene. The ID animals underwent the same iron-deficient diet regimen as the animals examined in the microarray analysis. Six of the twelve genes investigated in the original PCR analysis were randomly selected for verification, of which four were up-regulated and two were down-regulated.

Discussion

Microarray analysis provides the opportunity to study a broad spectrum of changes that occur in iron deficiency. In this study, two experimental groups of rats were investigated. One group, 21-day-old rats represented a state of brain development during which iron availability was compromised. This group permitted evaluation of on-going responses in the brain which could be adaptive (i.e. limiting processes because of the limited iron) or compensatory (i.e. increase in expression in an attempt to acquire more iron or other nutrients). The arrays in the six-month-old animals provided the opportunity to identify any long term consequences of the

early developmental iron deficiency. We observed changes in the expression of over 300 genes in the 21-day animals. The mRNA expression patterns in the 21-day animals indicate that multiple pathways throughout the brain are affected during iron deficiency. Because the array studies were performed during iron deficiency in the 21 day rats, these changes both reflect deficits directly due to the iron deficiency and possibly short-term adaptive changes to metabolic insufficiencies related to compromised iron availability. Contrastingly, the 6-month animals demonstrate the long-term, apparently irreversible changes resulting from the pre- and post-natal iron deficiency that are present even after five months of iron repletion. The changes in gene expression in the six month animals were not identified at the 21-day time period, suggesting that the gene changes at six months represent the chronic impact of iron-deficiency.

Iron Responsive Elements in Brain Iron Deficiency

There are two levels at which iron status could have a direct impact on gene expression levels. First, transcription factor activation could impact expression. There are no known transcription factors wherein the activity of the factor is directly related to the iron status of the factor. Secondly, a number of mRNAs are known to contain an iron responsive element (IRE) and/or be regulated by an iron regulatory protein (IRP). These genes are listed in Table 4.4. Of these, we identified the following as changed in the 21-day-old rats: transferrin receptor, divalent metal protein-1, and 5-aminolevulinate synthase. These will be discussed later. Eisenstein and Ross propose that the role of the iron regulatory proteins (IRPs) is not limited just to regulating iron metabolism proteins, but rather includes regulation of proteins involved in intermediary metabolism. As such, they argue that IRPs are critical to the adaptive response in iron deficiency (Eisenstein and Ross 2003). Iron regulatory proteins are cytosolic RNA-binding proteins that interact with specific small stem-loop structure elements in mRNA transcripts referred to as iron

response elements. As an example of the coordinated activity of IRPs on mRNAs containing an IRE, we present the post-transcriptional regulation model of ferritin and the transferrin receptor.

The H and L subunits of ferritin contain a single IRE near the 5' end, and in low-iron conditions, bound IRP blocks recruitment of the 40S subunit to the mRNA. Transferrin receptor mRNA contains five IREs in its 3' untranslated region (UTR). In the bound state, the IREs in TfR mRNA protect the mRNA from degradation, resulting in increased mRNA stability and enhanced TfR protein synthesis (Leibold and Munro 1988; Mullner and Kuhn 1988; Hentze and Kuhn 1996). TfR mRNA also contains a rapid-turnover determinant in the 3' UTR, such that in iron-sufficient states (when IRP is not bound) the mRNA is degraded starting at this site (Binder, Horowitz et al. 1994).

There exist two distinct IRPs, IRP1 and IRP2. The presence or absence of iron-sulfur clusters determines the functionality IRP1 (Emerit, Beaumont et al. 2001). When the cluster is present, as occurs in conditions of sufficient iron supply, IRP1 acts as an aconitase. In states of iron depletion, the cluster is absent, and IRP1 takes on the role of a high-affinity binding site for IREs. IRP2 does not contain an iron-sulfur cluster, and does not have aconitase activity. IRP2 is rapidly degraded under conditions of adequate iron supply (Kuhn 1998). Differences in the sequence and structure of the IRE stem region have an impact on IRE function. Stem region sequences of IREs are well conserved within IRE species (90% identical), but are less well conserved (35–90%) between IRE species. IRP1 and IRP2 prefer to bind to different IREs, depending on the structure of the IRE stem region (Ke, Sierzputowska-Gracz et al. 2000). The elegant system of IRP-IRE iron regulation allows for close control of cellular iron homeostasis by the iron molecule itself. The regulation of IRPs themselves is poorly understood. Some reports exist that IRP expression is responsive to oxygen status of the cell (Rouault and Klausner

1997). Dysfunction of the IRP system may result in neurodegenerative disease. For example, IRP/IRE interaction in the brain is disrupted in some individuals with Alzheimer's Disease (Connor, Menzies et al. 1992) and targeted deletion of the IRP-2 gene in mice may cause misregulation of iron metabolism and a neurodegenerative disease characterized by neuronal iron accumulation (LaVaute, Smith et al. 2001). A decrease in expression of IRP1 is under investigation as a cause of Restless Legs Syndrome (Connor, Wang et al. 2004).

In addition to the Tf receptor mRNA and ferritin mRNAs, there are other mRNAs that are known to contain an IRE sequence. DMT1 mRNA (identified in the gene array as solute carrier family 11 member 2, Slc11a2) was up-regulated in the 21-day animals. DMT1 is a proton symporter for ferrous iron and other divalent metal ions, and is responsible for iron uptake from the gut and transport from endosomes (Gruenheid, Cellier et al. 1995; Gunshin, Mackenzie et al. 1997). DMT1 has several mRNA splice variants, with only some containing an IRE-like element in the 3' UTR (Hubert and Hentze 2002). Though the DMT1 IRE binds to IRP1 (Gunshin, Allerson et al. 2001), it is not clear if binding is required for the iron-dependent changes in DMT1 mRNA expression, and thus the function of IRP in DMT1 regulation remains uncertain. DMT1 expression was changed in this study and the changes are discussed later in the context of other solute carriers.

FP mRNA has an IRE in its 5' UTR, and the IRE appears to bind IRP in *in vitro* studies (Abboud and Haile 2000; Donovan, Brownlie et al. 2000). In liver, iron regulation of FP is similar to ferritin, but in duodenum, FP expression regulation is opposite that of ferritin (Abboud and Haile 2000). In iron-deficient liver, FP mRNA translation is inhibited, and, conversely, injection of iron stimulates FP mRNA translation (Eisenstein and Ross 2003). In intestine, however, FP mRNA has a longer 5' UTR and the IRE is further from the 5' end (Abboud and

Haile 2000), possibly allowing the IRE to be ignored, as has been shown with synthetic constructs (Abboud and Haile 2000). FP was not identified in this study as altered although we have shown FP expression in brain (Clardy, Wang et al, submitted), but FP expression was unchanged in a rat model of compromised brain iron status (Burdo, Menzies et al. 2001).

Targets for IRP have been found also in the tricarboxylic acid (TCA) cycle and the electron-transport chain; an example is the TCA-cycle enzyme mitochondrial aconitase (m-acon). Expression of m-acon is regulated by iron in an IRP-dependent manner. Changes in m-acon levels in iron-deficient liver do not directly alter the functioning of the TCA-cycle, but are associated with an increase in the amount of radiolabeled citrate released from the mitochondria. Eisenstein et al propose that such findings support a hierarchy of function under iron-deficient conditions (Eisenstein and Ross 2003). IRE or IRE-like elements are also found in the mRNA encoding other proteins with critical roles in iron metabolism, including 5-aminolevulinate synthase (eALAS, ALAS2) (Bhasker, Burgiel et al. 1993; Melefors, Goossen et al. 1993). eALAS was up-regulated in the 21-day animals. Because IRE is found only in the erythroid isoform of ALAS, Eisenstein et al (Eisenstein and Ross 2003) suggest that IRPs are involved in the major daily exchange of iron between the erythron, the plasma, and the reticuloendothelial system.

Known Iron Transport Proteins, 21-Day Animals

Transferrin receptor was among the most significantly up-regulated genes in the 21-day rats. TfR up-regulation at the protein level is a consistent finding in models of iron deficiency for numerous cell types (Pinero, Li et al. 2000; Han, Day et al. 2003; Siddappa, Rao et al. 2003) but the mRNA expression is not always altered. An increase in TfR mRNA in our animals is an indication that the brains of these animals were iron-deficient (personal communication, JL

Beard). Neurons express TfR including during development (Bartlett, Li et al. 1991; Roskams and Connor 1992), and gestationally iron-deficient Sprague Dawley rats have increased neuronal expression of TfR protein in the hippocampus and the cerebral cortex (Siddappa, Rao et al. 2003). Moos et al. did not find an increase in Tf mRNA in medial habenular neurons in a rat iron-deficient model, which may be due to the specific nuclei investigated (Moos, Oates et al. 1999). On the other hand, Han et al. measured TfR RNA levels in rat brain, and found a regional increase in TfR RNA in iron deficiency, but the increase was only significant in striatum and the CA2 and CA3 regions of hippocampus (Han, Day et al. 2003). Additionally, the Han et al., study utilized a model of iron deficiency wherein the animals were iron sufficient until placed on an iron-deficient diet at rat postnatal day 21 for the next 6 weeks. The animal model in our study was significantly different, as the animals were iron-deficient prenatally through postnatal day 21. The timing of the iron deficiency in animal models suggests that the increase in Tf receptor mRNA is a typical response to brain iron deficiency. The inborn mechanism(s) governing the level and priority of regional brain iron requirements in early prenatal life are unclear, but certainly crucial, and the expression pattern of TfR mRNA may help elucidate the impact of these various models on iron status and hence metabolism.

Transferrin mRNA expression was significantly down-regulated in this study. In an acquired systemic iron deficiency, generally there is an increase in the level of Tf mRNA in the liver (Fleming, Migas et al. 2000; Han, Day et al. 2003). The contrast between the liver Tf RNA response and brain Tf RNA response in iron deficiency is not unique to this study. Han et al. (2003) examined both Tf mRNA and protein regionally throughout the brain in a different rat model of iron deficiency (Han, Day et al. 2003). In agreement with our studies, they found that Tf RNA content was decreased in iron-deficient animals in most brain regions. Contrastingly,

levels of Tf protein increased throughout the brain in iron-deficient animals, demonstrating an inverse correlation between Tf and its mRNA in iron deficiency, and suggesting that the translation efficiency of Tf RNA is increased or that Tf protein may be derived from tissue outside the brain. An increase in Tf transport into the brain in conditions of iron deficiency has been demonstrated (Crowe and Morgan 1992). Additionally, the Han study also measured liver Tf RNA and found the levels to be increased in the iron deficienct animals arguing against an increase in Tf translation efficiency within the brain to account for the increased Tf levels.

Cluster Analysis, 21 Day Animals

Myelin-related genes

The importance of myelin in development is underscored by its involvement in an array of neurological diseases, including leukodystrophies and multiple sclerosis. In the brain, the oligodendrocyte is the primary cell responsible for the formation of myelin. Hypomyelination is a consistent finding in iron deficiency (Larkin 1990; Beard, Wiesinger et al. 2003; Ortiz, Pasquini et al. 2004). Transferrin mRNA, which is down-regulated in our model, is normally found in oligodendrocytes and the choroid plexus (Aldred, Dickson et al. 1987; Espinosa de los Monteros, Kumar et al. 1990; Roskams and Connor 1992). Tf mRNA production and oligodendrocyte maturation are tightly coupled (Bartlett, Li et al. 1991).

In addition to Tf mRNA, several genes related to myelin were found to be down-regulated. Those genes include myelin and lymphocyte protein, myelin oligodendrocyte glycoprotein, myelin basic protein, myelin-associated oligodendrocytic basic protein, proteolipid protein, and peripheral myelin protein 22. Tf mRNA is, as mentioned, associated with oligodendrocyte maturation, and the myelin genes affected in our study impact on a number of

myelin processes. Myelin-associated oligodendrocytic basic protein (MOBP) mediates the later steps of myelin formation, possibly myelin compaction and myelin sheath maintenance (Holz, Schaeren-Wiemers et al. 1996). Myelin and lymphocyte protein (MAL) is an integral membrane protein that appears to be involved in myelin biogenesis and function and is critical in the maintenance of CNS paranodes, likely as a component in vesicular trafficking cycling between the Golgi complex and the apical plasma membrane (Schaeren-Wiemers, Bonnet et al. 2004). Myelin-oligodendrocyte glycoprotein (MOG) is also an integral membrane protein, and is a component of the compact myelin of the CNS, and is implicated in myelin stability, likely in completion and maintenance of the myelin sheath and in cell-cell communication. Reduced concentrations of MOG are observed in *jimpy* and *quaking* dysmyelinating mutant mice (Pham-Dinh, Mattei et al. 1993). Found exclusively in the CNS, MOG is localized on the surface of myelin and oligodendrocyte cytoplasmic membranes.

Myelin basic protein (MBP) also stabilizes the myelin membrane in the CNS. There are several isoforms of MBP; isoforms 4-13 are among the most abundant protein components of the myelin membrane in the CNS and they have a role in both its formation and integrity. The non-classic group of MBP isoforms (isoforms 1-3) may have a role in the developing brain before myelination, possibly as components of transcriptional complexes and/or in signaling pathways in neural cells (Pedraza, Fidler et al. 1997). The expression of MBP isoforms is developmentally regulated, with expression of the classic isoforms (isoforms 4-14, missing the first 134 amino acids) occurring later, likely as the oligodendrocytes approach terminal differentiation. Defects in MBP cause dysmyelinating diseases, as in *shiverer* (*shi*) and myelin-deficient (*mld*) mice. Both types of mice have decreased myelination in the CNS, tremors, and progressive convulsions. The *shiverer* mice express only isoform 2, while the *mld* mice have a reduction in

MBP (Shiota, Ikenaka et al. 1991). The consequences of these defective MBP animal models highlight the potential risks of decreased MBP expression. Our finding of reduced MBP RNA expression supports work by Beard et al. (2003) that reduced MBP in hindbrain in both pre-weaning and post-weaning rats iron-deficient rat models (Beard, Wiesinger et al. 2003).

Peripheral myelin protein 22 (PMP22) is an integral membrane protein found in the Schwann cells that mediates growth and peripheral myelin compaction. The human homolog gene duplication causes Charcot-Marie-Tooth 1A (CMT1A) neuropathy. A defect in PMP-22 is the cause of *trembler* (*tr*) phenotype in mice, which show a Schwann cell defect characterized by severe hypomyelination and Schwann cell proliferation throughout life.

The final myelin-related gene, proteolipid protein (PLP), is found in myelin but its cellular function remains obscure. Mutations of the X-chromosome-linked PLP gene can be lethal, as shown in the *jimpy* mouse and later in patients with Pelizaeus-Merzbacher disease. Phenotypically, these mutations include degeneration of oligodendrocytes and associated hypomyelination (Schneider, Montague et al. 1992).

Given the diverse and essential roles of all these genes in all stages of myelination, these data are highly suggestive of myelin deficiencies that occur at the mRNA level in the 21-day animals. The decrease in myelin related genes and Tf mRNA could reflect an adaptive response to the lack of a key nutrient that is required for metabolic support to maintain myelin. Given the neurological deficits associated with defective myelin, there may be an advantage to the system to delay myelination until nutrients become available. The signaling mechanism for inducing myelin onset may include iron release from microglia (Cheepsunthorn, Radov et al. 2001) which would be consistent with delayed myelination and iron deficiency. The decreased expression of myelin genes may also reflect a decreased number of oligodendrocytes. A decrease in the

number or maturation of oligodendrocytes would be consistent with the decrease in Tf mRNA and mRNAs for carbonic anhydrases (see Transition Metal Cluster in Table 4.2) seen in our study and the decrease in CNPase activity (a marker of oligodendrocyte metabolic activity) in iron deficiency reported by Beard et al. At this time the data do not allow us to rule out whether the decrease in myelin genes is related to fewer oligodendrocytes or decreased metabolic activity of a normal number of oligodendrocytes.

In our six-month-old rats using the same prenatal iron deficiency paradigm, the myelin genes were no longer altered, but there was a decrease in myelin and in PLP and MBP protein (Ortiz, Pasquini et al. 2004). These data suggest that the iron supplementation at weaning was sufficient to support normal gene expression, but that critical timing events between axons and oligodendrocytes may have been disrupted or metabolic compromises occurred that could not be completely corrected.

Solute Carrier Family

Almost 300 genes have been classified into 43 families of the solute carrier (SLC) family (Hediger, Romero et al. 2004). These transporters can be involved both in uptake and efflux in transcellular transport and include, for example, transporters for glutamate, glucose, bicarbonate, sodium, chloride, and amino acids (Hediger, Romero et al. 2004). Several members of the solute carrier family were identified among the significantly changed genes. Because of their diversity and importance in nutrient transport, solute carrier (SLC) proteins are studied extensively as a mechanism to increase drug absorption (Zhang, Knipp et al. 2002). Six genes in this cluster were up-regulated and two genes were down-regulated in the iron-deficient rats. Slc11a2 (DMT1) was up-regulated, as noted in the discussion of known iron transport proteins. DMT1 is

a transmembrane proton symporter not only for ferrous iron, but also for Mn^{2+}, Co^{2+}, and Cu^{2}. In rat brain, DMT1 is expressed in the striatal neurons, the thalamus, the cerebellum, and the ependyma, and it likely mediates iron export from the endosome to the cytosol. Our gene findings support the work of Erikson and colleagues, who found increased DMT1 protein in all iron-deficient rat brain regions examined (Erikson, Syversen et al. 2004). This study extends the investigation of DMT1 in iron deficiency to the gene level. The versatility of DMT1 and the other up-regulated solute carrier family members is significant in the context of metal transport in the iron-deficient state and signals a compensatory response to increase iron uptake. However, a side-effect of the increase in DMT1 may be to increase uptake of other metals that are not deficient in these animals (Erikson, Syversen et al. 2004). Therefore part of the advantage of a gene array study in this iron-deficient model may be to address the possibility that iron deficiency establishes an opportunity for neurotoxicity from other metals.

The up-regulated gene Slc16a1 (MCT1) codes for the proton-linked monocarboxylic acid transporter that catalyzes the transport across the plasma membrane of many monocarboxylates, including lactate and pyruvate, as well as ketone bodies. Baud et al (2003) studied the expression of the monocarboxylate transporters MCT1 in the rat brain and found that during the first postnatal week, MCT1 immunoreactivity extended to the vessel walls and to the developing astrocytes in the cortex. They proposed that the transient pattern of expression of MCTs throughout the perinatal period suggests a potential relationship with the maturation of the blood-brain barrier (BBB). In addition, Vannucci and Simpson (2003) suggest that the normal peak in MCT1 mRNA in the BBB during suckling and its later decline with maturation is directly related to a switch from a combination of glucose and ketone bodies to glucose as the predominant cerebral fuel. Glia, however, maintain constant MCT1 levels, implying a major role for these

proteins in transferring glycolytic intermediates during cerebral metabolism (Vannucci and Simpson 2003). MCT1 is up-regulated in our study at PND 21, which could suggest perturbed development of the BBB (Baud, Fayol et al. 2003) and altered timing of the switch from ketone bodies to glucose during development for metabolic support.

Another solute carrier, that is expressed in the BBB (and also the choroid plexus) is Slc21a5 (Reichel, Gao et al. 1999; Gao, Hagenbuch et al. 2000). The expression of this gene was decreased in iron deficiency. It transports digoxin and accepts the cyclic opioid pentapeptide [D-penicillamine2,5]enkephalin (DPDPE) as a substrate (Noe, Hagenbuch et al. 1997; Kakyo, Sakagami et al. 1999; Reichel, Gao et al. 1999), and may be involved in the transport of opioid peptides across the BBB in humans. The other gene in this cluster that was also down-regulated was Slc21a10 which was though to be a liver-specific. This gene is a sodium-independent transporter that mediates transport of a variety of compounds (Li, Hartley et al. 2002). Our findings suggest that there are significant alterations in transport mechanisms in the BBB as a result of iron deficiency.

Three solute carrier genes whose proteins are involved in neurotransmitter regulation were up-regulated. Slc18a2 (SVAT) is involved in the ATP-dependent vesicular transport of biogenic amine neurotransmitters, and was up-regulated by iron deficiency. SVAT pumps cytosolic monoamines (dopamine, norepinephrine, serotonin, and histamine) into synaptic vesicles. It can also transport N-methyl-4-phenylpyridinium (MPP+), a neurotoxin metabolite associated with Parkinson's disease that utilizes iron to induce apoptosis (Kalivendi, Kotamraju et al. 2003; Shang, Kotamraju et al. 2004). Slc6a4 (5HTT, 5HT transporter, Sert) is directly involved with serotonin, as it terminates the action of serotonin by reuptake into presynaptic terminals. Kaladhar and Narasinga (1982) found that in both moderate and severe forms of iron

deficiency, 5-HT uptake by brain synaptic vesicles is decreased (Kaladhar and Narasinga Rao 1982). Burhans et al. (2005) found that iron-deficient adult male rats had reduced 5-HT transporter binding in some brain regions, but females demonstrated increased 5-HT transporter binding. In our study, we included only males (Burhans, Dailey et al. 2005). These results indicate that iron deficiency disrupts serotonin homeostasis, but also indicates a disconnect between the mRNA expression and protein expression. Therefore, the serotoninergic system could be impacted by metabolic compromises due to iron deficiency that affect protein translation. The third up-regulated transporter in this group effecting neurotransmitters is Slc6a6, a sodium and chloride dependent taurine transporter that plays a role in neurotransmitter metabolism.

Finally, Slc7a1, a high-affinity, low capacity amino acid transporter involved in the transport of the cationic amino acids (arginine, lysine and ornithine) is up-regulated. The importance of this discussion on the effects of iron on these solute carriers is that the solute carrier (SLC) proteins have critical physiological roles in nutrient transport. The significant alterations shown here in their levels demonstrate how loss of iron availability can broadly impact nutrient transport (Zhang, Knipp et al. 2002). The gene array data strongly suggest that movement of ions and molecules between cells is compromised in the 21-day old iron-deficient animals.

Signal transduction

In the signal transduction cluster, some of the myelin-related genes appear because of their role in ionic insulation of neurons by glial cells. These are myelin and lymphocyte protein, myelin oligodendrocyte glycoprotein, myelin basic protein, and peripheral myelin protein 22.

Chimerin 2 was down-regulated by iron deficiency. Mizuno et al report that chimerin transcription is up-regulated after exposure to neurotrophins in cerebellar neurons (Mizuno, Yamashita et al. 2004). Chimerin 2 is important to regulating neurite outgrowth in relation to myelin signals (Mizuno, Yamashita et al. 2004). Fibroblast growth factor (FGF9), also known as glia-activating factor in the iron-deficient, is also down-regulated in this cluster. FGF9 is produced mainly by neurons and can act on FGF receptors (FGFR) and the major myelin proteins. In developing rat brain oligodendrocytes, for example, Cohen et al found that FGF-9 decreased levels of myelin proteins (Cohen and Chandross 2000). This down-regulation of both chimerin 2 and FGF9 suggests a mechanism for disrupting the timing of myelin-axon relationships. Furthermore, phosphatidylinositol 4-kinase, which is also involved in cell signaling and cytoskeleton function (Zambrzycka and Kacprzak 2003) is also down-regulated.

Three neurotransmitter receptors in the signal transduction cluster: dopamine receptor 1A, gamma-aminobutyric acid A receptor delta, and gamma-aminobutyric acid A receptor alpha 6 are down-regulated in the cluster. These data support research suggesting that early iron-deficiency can affect GABA and dopamine neurotransmitter metabolism (Taneja, Mishra et al. 1986; Youdim, Ben-Shachar et al. 1989; Erikson 2000). The neurotransmitter/neuromodulator, Galanin is also down-regulated in this cluster. The inhibitory effects of galanin on several forms of synaptic plasticity, including long-term potentiation, have been demonstrated in normal and transgenic animals (Xu, Zheng et al. 2005). A decrease in galanin thus represents less inhibition of synaptic plasticity, and coupled with the decreased expression of receptors involved in inhibition, suggests an overall state of decreased inhibition in the 21-day ID brain.

Two genes in this cluster that influence cytoskeletal function, afadin and phosphatidylinositol 4-kinase, are down-regulated. Afadin is a nectin- and actin-filament-

binding protein, and several of the nectins that afadin binds are involved in formation of synapses (Kakunaga, Ikeda et al. 2005), so a decrease in this gene expression could be consistent with the aforementioned loss of synaptic plasticity.

Channel/Pore class transporter & Alpha-type channel activity

This cluster contains several important types of transport channels, and reflects a broad-ranging down-regulation of ionic transport. Of the eleven genes in this cluster, nine are calcium, potassium, and chloride, or GABA channel subunits. Also down-regulated are the FXYD domain-containing ion transport regulator 1, the purinergic receptor P2X ligand-gated ion channel 4, and gap junction membrane channel protein beta 1 (gjb1), defects in which can cause the demyelinating form of X-linked Charcot-Marie-Tooth disease. These data further support the concept that cell to cell communication is compromised by iron deficiency.

DNA binding

All members of the DNA binding cluster were up-regulated in the iron-deficient rats. These genes included high mobility group box 1, high mobility group box 2, nuclease sensitive element binding protein 1 (Y-box binding protein 1 – inhibits protein synthesis), nuclear factor I/B, POU domain class 3 transcription factor 1, proliferating cell nuclear antigen, and paired box homeotic gene 6. The function of several of these genes is just beginning to be intensively investigated. Paired box homeotic gene 6 (PAX6), for example, has been shown to have an anti-proliferation function in cortical cell development by *in vivo* mouse studies (Estivill-Torrus, Pearson et al. 2002). In the case of the gene nuclear factor I/B, recent studies have defined a role for the NFI proteins as neuronal transcriptional regulators that participate in directing the

differentiation of cerebellar granule neurons via GABRA6; NFI proteins are required by GABRA6 for promoter activity and expression in neurons (Wang, Stock et al. 2004). As discussed earlier in the context of signal transduction, GABRA6 mRNA is down-regulated in iron deficiency, and up-regulation of Nfib mRNA expression may be a compensatory response to improve the efficiency of the NFI/GABRA6 directed neuronal differentiation. Nfib, specifically, is essential for brain development, and Nfib-deficient mice exhibit forebrain defects as well as defects in basilar pons and hippocampus formation (Steele-Perkins, Plachez et al. 2005). Based on current evidence, the up-regulation of at least the majority of these genes suggests slower replication of cells in the iron-deficient brain.

The up-regulation of some of these DNA binding genes also occurs in the setting of cell stress, particularly injury or inflammation. The identification of these genes therefore suggests that the iron-deficient brain is likely under stress. The up-regulated high-mobility group box 1 protein (HMGB1), for example, was recently discovered to be a crucial cytokine in mediating the response to injury and inflammation (Lotze and Tracey 2005; Tsung, Sahai et al. 2005). Inhibition of HMGB1 activity with antibody decreased liver damage, and addition of recombinant HMGB1 worsened damage. Proliferating cell nuclear antigen (PCNA) mediates recruitment of DNA methyltransferase (Dnmt1), which plays a direct role in the restoration of epigenetic information during DNA repair (Mortusewicz, Schermelleh et al. 2005) is also up-regulated by iron deficiency. The up-regulation of the DNA binding genes associated with cell stress supports the idea that impaired energy metabolism and transmitter synthesis makes iron-deficient neurons more vulnerable (Youdim and Ben-Shachar 1987; de Deungria, Rao et al. 2000). These data may be relevant to the cell stress markers seen in H-ferritin-deficient mice

that have compromised iron storage capacity and show increased evidence of stress (Thompson, Menzies et al. 2003).

Additional Genes identified as significantly changing expression in the 21-day old animals

The human kallikrein 6 gene (KLK6) encodes for a secreted serine protease, hK6, which is highly expressed in brain. Kallikrein was down-regulated in the 21-day iron-deficient animals. Previous reports have associated KLK6 with the pathogenesis of Alzheimer's disease. Zarghooni et al (2002) found that the brain of Alzheimer's disease (AD) patients contains significantly less KLK6 than the brain of non-affected individuals (Zarghooni, Soosaipillai et al. 2002). KLK6 (also known as neurosin) may be an aging-related protease, and a decreased concentration of neurosin may be a risk factor for developing AD in older humans. KLK6 has not been studied in the context of iron, but these data suggest a direct relationship. It is possible that the KLK6 findings in humans may be related to the iron deposition found in Alzheimer's disease (Jellinger, Paulus et al. 1990; Connor, Menzies et al. 1992).

Arachindonate 12-lipoxygenase (Alox12, 12-LOX) is significantly up-regulated in the 21-Day animals. 12-LOX has been identified as a facilitator of glutamate-induced cell death in neurons, and Khanna et al (2003) demonstrated that glutamate-induced 12-LOX activity can be blocked by nanomolar concentrations of a-tocotrienol (generically known as Vitamin E) (Khanna, Roy et al. 2003). In addition, they showed that 12-LOX-deficient primary cortical neurons are resistant to glutamate challenge, further supporting the role of 12-LOX in executing glutamate-induced neuronal death. Up-regulation of this gene suggests the potential for increased cell death, and supports the concept that iron-deficiency results in an environment of increased stress.

Membrane-associated guanylate kinase-interacting protein (MAGUIN) is expressed in neurons and is significantly up-regulated in the 21-Day animals. MAGUIN co-immunoprecipitates with the synaptic scaffolding proteins PSD-95/SAP90 and S-SCAM from rat crude synaptosome (Yao, Hata et al. 1999), suggesting that MAGUIN-1 may play an important role (with PSD-95/SAP90 and S-SCAM) in the assembly of synaptic junctions. Up-regulation of MAGUIN could be reflective of an effort to increase the number and/or stability of synaptic junctions.

Thyroid hormone responsive protein (THRSP) was the most significantly down-regulated gene from the 21-Day animals. Iron deficiency impairs thyroid hormone synthesis by reducing activity of heme-dependent thyroid peroxidase (Zimmermann and Kohrle 2002), and THRSP is known to be responsive to thyroid hormone. Iron deficiency is known to be associated with decreased thyroid hormone levels (Beard, Tobin et al. 1989). It is possible, therefore, that the decreased expression of THRSP mRNA is an adaptive response that will slow development under the current metabolic limitations imposed by decreased iron availability. A function of thyroid hormones in the developing brain is to provide a timing signal for the induction of differentiation and maturation programs, and inappropriate initiation of these timing events leads to asynchrony in developmental processes and a deleterious outcome (Anderson, Schoonover et al. 2003). Studies have historically suggested deficient development of the central nervous system in the absence of thyroid hormone - most notably reduced myelination (Balazs, Brooksbank et al. 1969; Balazs 1971; Rosman, Malone et al. 1972). In brain, THRSP is localized to neurons (Shah, Li et al. 1997). THRSP and thyroid hormone are likely involved in synaptic plasticity in hippocampal neurons (Tang, Ma et al. 2001). It is also relevant to note that Faivre et al. (1984) reported a dramatic decrease in microtubule numbers in Purkinje cells in

thyroid hormone-deficient states (Faivre 1984), and Aniello and colleagues (1991) also demonstrated a delay in the developmental expression patterns of various tubulin isotype mRNAs (Aniello, Couchie et al. 1991). The decrease in THRSP could be associated with the loss of synaptic plasticity discussed earlier, the decrease in myelin, and the decrease in cytoskeletal genes and myelin-axon interaction. The decrease in THRSP are perhaps also related to the down-regulation of CCT6A and TPM1 — and their resultant effects on tubulin and microtubules —observed in the 6-month animals, discussed in detail below. In the context of intervention strategies, a mechanism to increase THRSP may be essential before such strategies can be completely effective.

Six month Animals

Data from the 6-month old developmental iron-deficient rats is a striking contrast to the 21-day data: there are only five genes whose expression was changed and all are down-regulated. The significantly fewer gene changes at this later time point indicate that the adaptive and compensatory changes that occurred during the iron-deficient period were adequate in many areas but that iron repletion was not capable of overcoming all the development perturbations. The down-regulation of these genes that were still altered after five months of iron repletion represents decreased cytoskeletal stability, decreased nucleic acid translation, and decreased responsiveness to oxidative stress.

Two genes, DHX9 and H2AFY, are involved in nucleic acid translation. DHX9, or DEAD box polypeptide 9, is a putative RNA helicase. It is localized to the nucleus and is implicated in a number of cellular processes involving the alteration of RNA secondary structure, including translation initiation, nuclear and mitochondrial splicing, and ribosome and

spliceosome assembly (Zhang and Grosse 1994). It unwinds double-stranded DNA and RNA in a 3' to 5' direction and creates secondary structures capable of influencing RNA-binding proteins. H2A histone family, member Y (H2AFY) is a ubiquitous nuclear gene involved in DNA metabolism. Histones are responsible for the nucleosome structure of the chromosomal fiber in eukaryotes. Nucleosomes consist of approximately 146 bp of DNA wrapped around a histone octamer comprised of a pair of each of the four core histones (H2A, H2B, H3, and H4). Down-regulated expression of the DHXP and H2AFY genes suggests broad-ranging decreases in the efficiency of translation in the brain.

Two of the other genes identified in this array analysis, CCT6A and TPM1, are involved in cytoskeletal functioning and cell structure and stability. CCT6A encodes a cytoplasmic molecular chaperone that is member of the chaperonin containing TCP1 complex (CCT). This complex consists of two identical stacked rings, each containing eight different proteins, which fold in an ATP-dependent manner. The complex folds various proteins, including actin and tubulin - both of which are in turn also crucial to muscular and cytoskeletal stability. Tubulin is a component of the centrosome. Before microtubules can be nucleated, the tubulin must first be folded; this is achieved by the cytoplasmic chaperone complex TCP-1. A disruption in tubulin folding would have a negative impact on cell division and axonal transport. Actin, a protein abundant in many cells, especially muscle cells, significantly contributes to the cell's structure and motility. Actin can assemble microfilaments, interact with myosin to permit movement of the cell, or pinch the cell into two during cell division. Actin is found in neuronal growth cones and in synaptic complexes in the brain, specifically at the base of dendritic spines. Thus a disruption in actin could result in fewer branches on neurons and a decrease in synaptic efficacy associated with changes in dendritic spine configuration.

A common mechanism is likely involved in both the formation of dendritic spines and the structural plasticity at mature synapses, and that common mechanism is centered on dynamic actin filaments (Matus 2000). Fernandez-Valle et al (1997) found that disrupting actin polymerization with cytochalasin D inhibited myelination of Schwann cell/Neuron co-cultures, suggesting that filamentous actin is needed during SC differentiation for both cell shapes changes as well as expression of myelin-specific mRNAs (Fernandez-Valle, Gorman et al. 1997). Dendritic spines are comprised of two major structural elements: postsynaptic densities (PSD) and actin cytoskeletons. Takahashi et al (2003) demonstrated that synaptic clustering of drebrin, an actin-binding protein known, regulates spine morphogenesis. Further, suppression of drebrin up-regulation by antisense nucleotides attenuated synaptic clustering of PSD-95 and clustering of drebrin and filamentous actin (Takahashi, Sekino et al. 2003). Learning and memory deficits associated with early iron deficiency persist beyond development despite iron repletion (Felt and Lozoff 1996; Kwik-Uribe, Gietzen et al. 2000; Youdim and Yehuda 2000). A possible mechanism for the long-term deficits is provided by dendritic analysis of the hippocampus in iron-deficient rats (Rao, de Ungria et al. 1999; Rao, Tkac et al. 2003) that show decreased dendritic branching in the iron-deficient rats. Additionally, Georgieff and colleagues specifically found that severe perinatal iron deficiency disrupted hippocampal apical dendritic growth and branching patterns as indexed by microtubule associated protein-2 (MAP-2) when examined early in life (Jorgenson, Wobken et al. 2003). These data are consistent with our gene array studies that predict a loss of dendritic profiles could be found in iron-deficient brains.

Cytoskeleton actin filaments are stabilized by the ubiquitous protein Tropomyosin 1 (TPM1). TPM1 binds to actin filaments in muscle and non-muscle cells and plays a role in the regulation of striated muscle contraction (along with the troponin complex). The observed

decreases in CCT6A and TPM1 in the brains of the six month animals is suggestive of long-term damage to the cytoskeletal infrastructure of neurons and possibly the glial cells — a particularly devastating finding in the brain, where such proteins (and the affected downstream proteins) are necessary to form interneuronal connections. Again, these findings provide further support and potential precipitating mechanisms for the long-term deficits of iron deficiency.

Tropomyosins (TMs) are actin binding proteins that can be made more diverse by alternative RNA splicing. TMBr-1 and TMBr-3, for example, are two brain-specific isoforms of TPM1 in rat generated by alternative splicing. TMBr-3 appears embryonically at 16 days of gestation throughout the brain, while TMBr-1 does not appear until 20 days after birth and is expressed only in areas primarily derived from the prosencephalon (Stamm, Casper et al. 1993). TMBr-1 and TMBr-3 are expressed relatively late in development, with their levels remaining at a constant level in the normal adult. Further, TMBr-1 has been shown to have the strongest expression in areas of the brain thought to have the greatest plasticity after birth. Such patterns suggest a specialized role of these isoforms in nervous system development and plasticity. The function of TMs in non-muscle cells is not yet clear, but is certainly distinct from their function in muscle cells, where TMs function in association with the troponin complex to regulate calcium-sensitive actin and myosin interaction. Interestingly, TM expression is changed in some neurodegenerative diseases, including Parkinson's and Alzheimer's diseases (Galloway, Mulvihill et al. 1990; Galloway and Perry 1991). Stamm et al suggest that TMBr-1 and TMBr-3 may play a role in process outgrowth and the formation of dendritic spines and synapses, as TMs have been shown to be present in all of these structures (Stamm, Casper et al. 1993). Additionally, the likely weaker binding properties of the two isoforms may allow for the

dynamic cytoskeletal changes necessary for adult plasticity. The gene array data, both at the six-month and 21-day-old groups suggest plasticity is compromised in the iron-deficient brain.

The fifth down-regulated gene, proteasome subunit beta type 5 (PSMB5), is a member of the proteasome B-type family that is a 20S core subunit in the proteasome. Proteasomes degrade oxidized, damaged, or misfolded proteins, thus promoting cell survival. Proteasomes are both cytoplasmic and nuclear and are distributed throughout eukaryotic cells. They cleave peptides in an ATP/ubiquitin-dependent process in a non-lysosomal pathway. Some neurodegenerative diseases and age-related disorders are associated with reduced proteasome activity. Kwak et al (2003) showed that the expression of most subunits of the 20S proteasome were enhanced up to threefold in the livers of mice following treatment with dithiolethiones, which act as indirect antioxidants (Kwak, Wakabayashi et al. 2003). A gene known as Nrf2 is a central molecular target of indirect antioxidants, and they demonstrated that the genes forming the 26S proteasome complex (of which PSMB5 and the 20s proteasome are a part) are regulated by Nrf2. Promoter activity of PSMB5 increased with either Nrf2 over-expression or treatment with antioxidants in mouse embryonic fibroblasts, and they identified antioxidant response elements in the promoter of PSMB5 that controlled these responses. Induction of this protective pathway provides a means for cells to survive conditions of stress. We observe down-regulation of the PSMB5 gene in our six month animals, suggesting less effective protein degradation compared to those animals who did not suffer the iron-deficient insult early in life.

Kabashi et al (2004) found that the level of 20S proteasome was reduced in lumbar spine motor neurons of transgenic SOD-1 mice relative to the surrounding neuropil, suggesting that impaired proteasomal function is an early event in amyotrophic lateral sclerosis (ALS) contributing to its pathology (Kabashi, Agar et al. 2004). Studies of the *trembler* mouse model

of Charcot-Marie-Tooth disease Type 1A also suggested a role for impaired proteasome activity (Fortun, Li et al. 2005). Aggregates containing ubiquitin and peripheral myelin protein 22 (PMP22) were found in neuropathy nerves, suggesting a mechanism by which defective degradation of Schwann cell proteins could contribute to PMP22 neuropathies

Alternatively, the down-regulation of PSMB5 could reflect an attempt to delay or prevent degradation in a stressful environment. MacInnis and Campenot (2005) suggest that the proteasome does not function as an effector of protein degradation during axonal degeneration, but rather it regulates the signaling pathways that control axonal survival or degeneration (MacInnis and Campenot 2005). They treated transected distal axons from rat sympathetic neurons with an inhibitor of proteasome activity, and found that it preserved axonal mitochondrial function over the tested 24 hours. The protected axons demonstrated persistent Erk1/2 phosphorylation, which, upon inhibition of MEK activity, restored axonal degeneration. Regardless of the exact role of PSMB5 in the six month animals, decreased proteasomal function could increase cell vulnerability.

Conclusion

The inability of normal iron in the diet beginning at weaning to reverse changes in the brain that affect cytoskeletal stability and synaptic function reinforce the importance of early intervention in iron deficiency. The gene arrays in the 21 days old rats identified a set of responses that could be characterized as adaptive to the existing state of iron deficiency and another set of responses that could be characterized as compensatory. The compensatory response (defined as changes in gene expression that would be aimed at normalizing iron status) consists of elevated Tf receptor mRNA and increase in DMT1. The adaptive responses, such as

decreased replication of cells, decreased myelin gene expression, decreased signal transduction pathway genes etc. indicate changes in gene expression that were due to the altered iron status. These adaptive responses could be returned to normal if iron repletion occurs in a sufficient and timely manner. The gene array data from the six-month-old animals suggests that most of the adaptive responses were effective. Some long term changes in gene expression where identified that could be related to the adaptive responses in the 21-day-old rats. For example, the cytoskeletal and synaptic plasticity deficits in the adult brain suggested by down regulation of tropomyosins and CCT6A could be related to the altered gene expression patterns associated with signal transduction and myelin proteins. In addition, a decrease in the integrity of this relationship may account for the myelin deficits seen in the six-month-old animals (Ortiz, Pasquini et al. 2004) despite the absence of changes in myelin gene profiles.

Embedded in the interpretation of our data is that the adaptive responses are likely to be dependent upon the developmental age at which the iron deficiency is encountered and the length of time that the animals are exposed to iron deficiency. At some point, if the adaptive responses are allowed to persist, the brain develops under the influence of these responses which should result in compromised function. The specific implications of the down-regulated genes, however, must be considered: metabolically, there is slowed translation and slowed degradation; structurally, there is cytoskeletal insufficiency. Such findings are remarkable in the context of such a long recovery time, and are suggestive of the irreversibility of an early iron insult. These data provide novel evidence for the behavioral and neurological deficits that are associated with developmental iron deficiency and identify key areas in the early development iron deficiency model that can be targeted to minimize the long-term consequences.

Table 4.1. Fifty most significantly changed genes from the 21-day rat group. This table lists 50 most significantly changed genes — 25 up-regulated and 25 down-regulated — from over 300 significantly genes in the 21-day rat group. Both the fold change and the q-value are listed, as the combination of these two values was considered in determining significance.

Table 4.1

	GENE NAME	Symbol	Fold Change	q-value (%)
UP-REGULATED				
L06040_s_at	arachidonate 12-lipoxygenase	Alox12	3.38871	0.83181
rc_AI237007_at	electron-transferring-flavoprotein dehydrogenase	Etfdh	2.51510	0.83181
M58040_at	transferrin receptor	Tfrc	2.39091	0.83181
AF102853_at	membrane-associated guanylate kinase-interacting protein	LOC59322	1.87739	0.83181
AB011679_at	tubulin, beta 5	Tubb5	1.87635	0.83181
rc_AA900769_s_at	smooth muscle alpha-actin	Acta2	1.84847	1.37095
rc_AA926149_g_at	catalase	Cat	1.84026	0.83181
X60767mRNA_s_at	cell division cycle 2 homolog A (S. pombe)	Cdc2a	1.67649	0.83181
rc_AI008836_s_at	high mobility group box 2	Hmgb2	1.66875	0.83181
AF022083_s_at	guanine nucleotide binding protein, beta 1	Gnb1	1.61726	0.83181
X03369_s_at	Rat mRNA for beta-tubulin T beta15	---	1.58431	0.83181
rc_AA899854_at	topoisomerase (DNA) 2 alpha	Top2a	1.57366	3.94082
rc_AA892390_s_at	solute carrier family 11 member 2	Slc11a2	1.56078	0.83181
D86642_at	FK506 binding protein 1b	Fkbp1b	1.55962	0.83181
rc_AA925762_at	Rattus norvegicus similar to Myristoylated alanine-rich C-kinase substrate (MARCKS), mRNA	---	1.55113	0.83181
rc_AI639294_at	Rattus norvegicus similar to protein ref (H.sapiens) KIAA0275 gene product	---	1.54511	2.90318
U75405UTR#1_f_at	collagen, type 1, alpha 1	Col1a1	1.54275	4.87630
rc_AA997865_at	thymosin beta-like protein	LOC286978	1.52727	0.83181
X13016_at	CD48 antigen	Cd48	1.51357	0.83181
M58364_at	GTP cyclohydrolase 1	Gch	1.50412	0.83181
rc_AI145680_s_at	solute carrier family 16, member 1	Slc16a1	1.50408	2.00860
D86297_at	aminolevulinic acid synthase 2	Alas2	1.49073	0.83181
U93306_at	kinase insert domain protein receptor	Kdr	1.48794	2.90318
AB000362_at	cold inducible RNA-binding protein	Cirbp	1.45745	4.87630
M64755_at	cysteine-sulfinate decarboxylase	Csad	1.45583	2.90318
DOWN-REGULATED				
K01934mRNA#2_at	thyroid hormone responsive protein	Thrsp	0.39906	0.83181
U31866_g_at	Rattus norvegicus Nclone10 mRNA	---	0.47015	0.83181
D38380_g_at	Transferrin	Tf	0.49119	0.83181
U31367_at	myelin and lymphocyte protein	Mal	0.51875	0.83181
AF016269_at	kallikrein 6	Klk6	0.53451	0.83181
AB003726_at	homer, neuronal immediate early gene, 1	Homer1	0.53698	0.83181
D28111_at	myelin-associated oligodendrocytic basic protein	Mobp	0.53918	0.83181
X55572_at	apolipoprotein D	Apod	0.54264	0.83181
rc_AI639532_at	Rat troponin-c mRNA	---	0.55172	0.83181
L21995_s_at	myelin oligodendrocyte glycoprotein	Mog	0.61224	0.83181
X60351cds_s_at	crystallin, alpha B	Cryab	0.61292	0.83181
D90401_at	afadin	Af6	0.63107	1.37095
U48828_g_at	Rattus norvegicus retroviral-like ovarian specific transcript 30-1 mRNA	---	0.63803	1.37095
X15512_at	apolipoprotein C-	Apoc1	0.64339	0.83181
X58294_at	carbonic anhydrase 2	Ca2	0.64519	0.83181
AF081196_at	RAS guanyl releasing protein 1	Rasgrp1	0.64721	1.37095
AF026529_s_at	stathmin-like 4	Stmn4	0.64929	0.83181
rc_AI639465_r_at	ring finger protein 28	Rnf28	0.65888	0.83181
rc_AA892775_at	lysozyme	Lyz	0.65940	1.37095
AF091563_r_at	olfactory receptor	LOC309574	0.66307	0.83181
H32189_s_at	glutathione S-transferase, mu 1	Gstm1	0.66508	0.83181
L03201_at	cathepsin S	Ctss	0.66680	0.83181
L33894_at	deoxycytidine kinase	Dck	0.67197	3.94082
rc_AI228548_g_at	Rattus norvegicus similar to S-100 protein, alpha chain (LOC295214), mRNA	---	0.67279	0.83181
D84479_at	Rat PMSG-induced ovarian mRNA, 3'sequence, N1	---	0.67470	3.78031

Table 4.2. Cluster Analysis. Each gene, symbol, and biological process is displayed within each of 6 clusters.

Table 4.2

Symbol	NAME	Biological Process
signal transduction - 13 genes		
Mal	myelin and lymphocyte protein	ionic insulation of neurons by glial cells, intracellular protein transport
Mog	Myelin oligodendrocyte glycoprotein	ionic insulation of neurons by glial cells
Af6	afadin	neuropeptide signaling pathway
Mbp	Myelin basic protein	ionic insulation of neurons by glial cells
Drd1a	dopamine receptor 1A	G-protein coupled receptor protein signaling pathway
Chn2	chimerin (chimaerin) 2	intracellular signaling cascade
Pik4cb	phosphatidylinositol 4-kinase	signal transduction
Gabrd	gamma-aminobutyric acid A receptor, delta	synaptic transmission; gamma-aminobutyric acid signaling pathway; chloride transport
Gal	galanin	neuropeptide signaling pathway
---	gamma-aminobutyric acid A receptor, alpha 6	---
Dlgh1	discs, large homolog 1 (Drosophila)	intracellular signaling cascade
Pmp22	peripheral myelin protein 22	ionic insulation of neurons by glial cells; cell cycle arrest
Fgf9	fibroblast growth factor 9	signal transduction
channel/pore class transporter & ion channel activity - 11 genes		
P2rx4	purinergic receptor P2X, ligand-gated ion channel, 4	ion transport
Clcn4-2	putative chloride channel (similar to Mm Clcn4-2)	---
Kcnab1	potassium voltage gated channel, shaker related subfamily, beta member 1	potassium ion transport
Fxyd1	FXYD domain-containing ion transport regulator 1	muscle contraction; calcium ion homeostasis
Scn1a	sodium channel, voltage-gated, type 1, alpha polypeptide	---
Gabrd	gamma-aminobutyric acid A receptor, delta	synaptic transmission; gamma-aminobutyric acid signaling pathway; chloride transport
Kv8.1	neuronal potassium channel alpha subunit	---
Cacna2d1	calcium channel, voltage-dependent, alpha2/delta subunit 1	ion transport
---	gamma-aminobutyric acid A receptor, alpha 6	---
Kcnd3	potassium voltage gated channel, Shal-related family, member 3	potassium ion transport
Gjb1	gap junction membrane channel protein beta 1	cell-cell signaling
DNA binding - 8 genes		
Hmgb2	high mobility group box 2	---
Nsep1	nuclease sensitive element binding protein 1	regulation of transcription, DNA-dependent
Nfib	nuclear factor I/B	regulation of transcription, DNA-dependent; DNA replication
Pou3f1	POU domain, class 3, transcription factor 1	regulation of transcription
Pcna	Proliferating cell nuclear antigen	---
Hmgb1	high mobility group box 1	regulation of transcription, DNA-dependent; DNA packaging; chromosome organization and biogenesis (sensu Eukarya)
Pax6	Paired box homeotic gene 6	development; regulation of transcription, DNA-dependent; eye morphogenesis (sensu Drosophila)
Hes1	hairy and enhancer of split 1 (Drosophila)	regulation of transcription, DNA-dependent
transition metal ion binding - 3 genes		
Tf	Transferrin	---
Ca2	carbonic anhydrase 2	---
Ca4	carbonic anhydrase 4	one-carbon compound metabolism
myelin related - 6 genes		
Mobp	myelin-associated oligodendrocytic basic protein	---
Mal	myelin and lymphocyte protein	ionic insulation of neurons by glial cells; intracellular protein transport
Mog	Myelin oligodendrocyte glycoprotein	ionic insulation of neurons by glial cells
Mbp	Myelin basic protein	ionic insulation of neurons by glial cells
Plp	proteolipid protein	nerve ensheathment
Pmp22	peripheral myelin protein 22	ionic insulation of neurons by glial cells; cell cycle arrest
solute carrier family - 8 genes		
Slc11a2	solute carrier family 11 member 2	---
Slc16a1	solute carrier family 16, member 1	transport;
Slc18a2	solute carrier family 18, member 2	transport
Slc6a6	solute carrier family 6, member 6	neurotransmitter transport; neurotransmitter secretion
Slc7a1	solute carrier family 7, member 1	transport; amino acid transport
Slc6a4	solute carrier family 6, member 4	neurotransmitter transport; neurotransmitter secretion
Slc21a5	solute carrier family 21 , member 5	organic anion transporter
Slc25a10	solute carrier family 25 member 10	mitochondrial carrier; dicarboxylate transporter

Table 4.3. All significantly changed genes in the 21-day animals. This table lists the 334 significantly changed genes in the 21-day rats. All genes have an FDR of <0.05 (the expected proportion of false positive genes among all genes that are considered significant). Each gene title, name, symbol, and ID is listed, as well as the fold change and q-value.

Table 4.3

Title	Symbol	Gene Name	Gene ID	Fold Change	q-value (%)
UP-REGULATED GENES					
arachidonate 12-lipoxygenase	**Alox12**	L06040_s_at	NM_031010	3.38871	0.831810561
arachidonate 12-lipoxygenase	**Alox12**	S69383_at	NM_031010	3.11209	0.831810561
Rattus norvegicus transcribed sequences	**---**	rc_AA866443_at	---	2.71356	0.831810561
electron-transferring-flavoprotein dehydrogenase	**Etfdh**	rc_AI237007_at	---	2.51510	0.831810561
transferrin receptor	**Tfrc**	M58040_at	---	2.39091	0.831810561
Rattus norvegicus transcribed sequences	**---**	rc_AA893172_at	---	2.14911	0.831810561
---	**---**	M93257_s_at	---	1.88482	3.940821135
membrane-associated guanylate kinase-interacting protein	**LOC59322**	AF102853_at	NM_021686	1.87739	0.831810561
tubulin, beta 5	**Tubb5**	AB011679_at	NM_173102	1.87635	0.831810561
smooth muscle alpha-actin	**Acta2**	rc_AA900769_s_at	---	1.84847	1.370947035
catalase	**Cat**	rc_AA926149_g_at	NM_012520	1.84026	0.831810561
cell division cycle 2 homolog A (S. pombe)	**Cdc2a**	X60767mRNA_s_at	NM_019296	1.67649	0.831810561
high mobility group box 2	**Hmgb2**	rc_AI008836_s_at	NM_017187	1.66875	0.831810561
guanine nucleotide binding protein, beta 1	**Gnb1**	AF022083_s_at	NM_030987	1.61726	0.831810561
Rat mRNA for beta-tubulin T beta15	**---**	X03369_s_at	---	1.58431	0.831810561
topoisomerase (DNA) 2 alpha	**Top2a**	rc_AA899854_at	---	1.57366	3.940821135
---	**---**	X62951mRNA_s_at	---	1.57265	0.831810561
solute carrier family 11 member 2	**Slc11a2**	rc_AA892390_s_at	NM_013173	1.56078	0.831810561
FK506 binding protein 1b	**Fkbp1b**	D86642_at	NM_022675	1.55962	0.831810561
Rattus norvegicus similar to Myristoylated alanine-rich C-kinase substrate (MARCKS) (LOC294446), mRNA	**---**	rc_AA925762_at	---	1.55113	0.831810561
Rattus norvegicus transcribed sequence with weak similarity to protein ref:NP_055582.1 (H.sapiens) KIAA0275 gene product [Homo sapiens]	**---**	rc_AI639294_at	---	1.54511	2.903181957
collagen, type 1, alpha 1	**Col1a1**	U75405UTR#1_f_at	---	1.54275	4.876302628
thymosin beta-like protein	**LOC286978**	rc_AA997865_at	NM_173313	1.52727	0.831810561
---	**---**	AF034899_r_at	---	1.52307	1.370947035
CD48 antigen	**Cd48**	X13016_at	NM_139103	1.51357	0.831810561
GTP cyclohydrolase 1	**Gch**	M58364_at	NM_024356	1.50412	0.831810561

solute carrier family 16, member 1	**Slc16a1**	rc_AI145680_s_at	NM_012716	1.50408	2.008596819
aminolevulinic acid synthase 2	**Alas2**	D86297_at	NM_013197	1.49073	0.831810561
collagen, type 1, alpha 1	**Col1a1**	M27207mRNA_s_at	---	1.48835	4.876302628
kinase insert domain protein receptor	**Kdr**	U93306_at	NM_013062	1.48794	2.903181957
Rattus norvegicus transcribed sequence with moderate similarity to protein pir:I37421 (H.sapiens) I37421 glutaminyl-peptide cyclotransferase (EC 2.3.2.5) - human	**---**	rc_AA859661_at	---	1.46779	0.831810561
Rattus norvegicus transcribed sequences	**---**	rc_AA875126_g_at	---	1.46017	0.831810561
cold inducible RNA-binding protein	**Cirbp**	AB000362_at	NM_031147	1.45745	4.876302628
cysteine-sulfinate decarboxylase	**Csad**	M64755_at	NM_021750	1.45583	2.903181957
fatty acid Coenzyme A ligase, long chain 4	**Facl4**	D85189_at	NM_053623	1.45503	1.370947035
high mobility group box 2	**Hmgb2**	rc_AA996401_s_at	NM_017187	1.44818	0.831810561
guanine nucleotide binding protein gamma subunit 11	**Gng11**	rc_AA860043_at	NM_022396	1.44635	2.008596819
cyclin-dependent kinase 4	**Cdk4**	L11007_at	NM_053593	1.42897	2.008596819
tissue factor pathway inhibitor	**Tfpi**	D10926_s_at	NM_017200	1.42397	0.831810561
Rattus norvegicus transcribed sequence with moderate similarity to protein pdb:1LBG (E. coli) B Chain B, Lactose Operon Repressor Bound To 21-Base Pair Symmetric Operator Dna, Alpha Carbons Only	**---**	rc_AI639203_at	---	1.42285	0.831810561
cd36 antigen	**Cd36**	AF072411_g_at	NM_031561	1.41508	2.903181957
lipocalin 2	**Lcn2**	rc_AA946503_at	NM_130741	1.41052	0.831810561
---	**---**	S83025_s_at	---	1.40337	0.831810561
carnitine O-octanoyltransferase	**Crot**	U26033_at	NM_031987	1.40241	0.831810561
Rattus norvegicus clone D920 intestinal epithelium proliferating cell-associated mRNA sequence	**---**	U21719mRNA_s_at	---	1.40037	3.940821135
TGFB inducible early growth response	**Tieg**	rc_AI172476_at	NM_031135	1.39376	3.780313527
Kruppel-like factor 4 (gut)	**Klf4**	L26292_g_at	NM_053713	1.38722	2.435234865
topoisomerase (DNA) 2 alpha	**Top2a**	D14045_s_at	---	1.38641	0.831810561
v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	**Ets1**	L20681_at	NM_012555	1.38250	4.876302628
Rattus norvegicus transcribed sequence with moderate similarity to protein sp:P00722 (E. coli) BGAL_ECOLI Beta-galactosidase (Lactase)	**---**	rc_AA799751_at	---	1.37841	0.831810561
Rattus norvegicus similar to replication protein A3 (LOC288727), mRNA	**---**	rc_AI171243_at	---	1.37818	4.876302628
high mobility group box 2	**Hmgb2**	D84418_s_at	NM_017187	1.37812	0.831810561
nuclease sensitive element binding protein 1	**Nsep1**	D13309_s_at	NM_031563	1.37323	0.831810561
solute carrier family 18, member 2	**Slc18a2**	L00603_at	NM_013031	1.37097	4.266201283
Rattus norvegicus transcribed sequence with weak similarity to protein sp:Q99536 (H.sapiens) VAT1_HUMAN Synaptic vesicle membrane protein VAT-1 homolog	**---**	rc_AA875639_at	---	1.37095	2.435234865
histamine N-methyltransferase	**Hnmt**	S82579_s_at	NM_031044	1.37068	3.940821135
high mobility group box 2	**Hmgb2**	D84418_r_at	NM_017187	1.36975	1.370947035
ribosomal protein L27	**Rpl27**	rc_AI176589_at	NM_022514	1.36464	0.831810561
Rattus norvegicus transcribed sequence with strong similarity to protein ref:NP_004042.1 (H.sapiens) 3-hydroxybutyrate dehydrogenase precursor; (R)-3-hydroxybutyrate dehydrogenase [Homo sapiens]	**---**	rc_AA817846_at	---	1.35981	4.876302628
nitric oxide synthase 3, endothelial cell	**Nos3**	AJ011116_at	---	1.35870	2.903181957
dihydropyrimidinase-like 3	**Crmp4**	U52104_at	NM_012934	1.35597	2.435234865
parathyroid hormone receptor 1	**Pthr1**	M77184_i_at	NM_020073	1.34793	2.903181957

Rattus norvegicus transcribed sequences	**---**	rc_AA894210_at	---	1.34657	4.266201283
neuronatin	**Nnat**	U08290_at	NM_053601	1.34477	2.008596819
solute carrier family 6, member 6	**Slc6a6**	M96601_at	NM_017206	1.34394	3.940821135
POU domain, class 3, transcription factor 1	**Pou3f1**	M72711_at	NM_138838	1.34375	2.903181957
solute carrier family 16, member 1	**Slc16a1**	D63834_at	NM_012716	1.33983	0.831810561
proline-rich proteoglycan 2	**Prpg2**	L17318_at	NM_172065	1.33929	2.903181957
RAB11a, member RAS oncogene family	**Rab11a**	M75153_g_at	NM_031152	1.33755	3.940821135
cell division cycle 42 homolog (S. cerevisiae)	**Cdc42**	rc_AA925473_at	NM_171994	1.33461	3.940821135
nuclear factor I/B	**Nfib**	rc_AI176488_at	---	1.33438	2.435234865
nuclear factor I/X	**Nfix**	AB012235_at	---	1.33244	3.780313527
fatty acid binding protein 7	**Fabp7**	U02096_at	NM_030832	1.33049	2.008596819
methionine aminopeptidase 2	**Metap2**	L10652_g_at	NM_022539	1.32941	2.903181957
Rattus norvegicus similar to Tde1 protein (LOC296350), mRNA	**---**	rc_AA799641_g_at	---	1.32728	2.903181957
hypocretin receptor 1	**Hcrtr1**	AF041244_at	NM_013064	1.32437	2.435234865
myristoylated alanine rich protein kinase C substrate	**Marcks**	rc_AA899253_at	---	1.32349	2.903181957
amyloid beta (A4) precursor protein-binding, family A, APBA1: amyloid beta (A4) precursor protein-binding, family A, member 1 (X11)	**Apba1**	AF029105_at	NM_031779	1.32122	3.940821135
myosin regulatory light chain	**Mrlcb**	X54617mRNA_s_at	NM_017343	1.31934	2.435234865
paired box gene 6	**Pax6**	S74393_s_at	NM_013001	1.31871	4.876302628
solute carrier family 11 member 2	**Slc11a2**	AF008439_g_at	NM_013173	1.31643	2.008596819
rabphilin 3A-like (without C2 domains)	**Rph3al**	AF022774_g_at	NM_133591	1.31547	2.903181957
cyclin B1	**Ccnb1**	rc_AA998164_s_at	NM_171991	1.31417	2.903181957
Rattus norvegicus transcribed sequences	**---**	rc_AA799764_at	---	1.31083	4.876302628
agrin	**Agrn**	M64780_at	NM_175754	1.31033	2.435234865
integrin alpha M	**Itgam**	U59801_at	NM_012711	1.30846	2.435234865
agrin	**Agrn**	M64780_g_at	NM_175754	1.30844	3.940821135
thymosin, beta 10	**Tmsb10**	M58404_at	NM_021261	1.30837	2.903181957
Rattus norvegicus transcribed sequences	**---**	rc_AA893663_at	---	1.30783	2.903181957
tyrosine 3-monooxgenase/tryptophan 5-monooxgenase activation protein, gamma polypeptide	**Ywhag**	S55305_s_at	NM_019376	1.30555	0.831810561
TGFB inducible early growth response	**Tieg**	rc_AI071299_at	NM_031135	1.30535	3.940821135
high mobility group box 1	**Hmgb1**	M64986_at	NM_012963	1.30395	3.940821135
neurotrophin-3 (HDNF/NT-3)	**Ntf3**	M34643_at	NM_031073	1.30317	2.903181957
nuclear protein E3-3	**LOC56769**	U95162_at	NM_020080	1.30064	4.876302628
high mobility group box 2	**Hmgb2**	rc_AI044390_s_at	NM_017187	1.29966	2.903181957
ras-related protein rab10	**Rab10**	rc_AI230406_at	NM_017359	1.29807	3.940821135
Rattus norvegicus transcribed sequences	**---**	rc_AI639359_at	---	1.29613	2.008596819
cytochrome P450, subfamily 2G, polypeptide 1	**Cyp2g1**	M31931cds_s_at	---	1.29574	2.903181957
mitogen activated protein kinase kinase kinase 1	**Map3k1**	U48596_at	NM_053887	1.29104	2.903181957
---	**---**	K00750exon#2-3_g_at	---	1.28964	2.435234865
---	**---**	X65190mRNA_s_at	---	1.28905	2.903181957
solute carrier family 7, member 1	**Slc7a1**	rc_AA957917_s_at	NM_013111	1.28611	4.266201283
Rattus norvegicus similar to dimethylarginine dimethylaminohydrolase 1; NG,NG dimethylarginine dimethylaminohydrolase (LOC365956), mRNA	**---**	rc_AI058941_s_at	---	1.28423	3.940821135
phosphodiesterase 7A	**Pde7a**	U77880_at	---	1.28365	3.940821135
solute carrier family 6, member 4	**Slc6a4**	X63253cds_s_at	NM_013034	1.28237	3.940821135

collapsin response mediator protein 1	**Crmp1**	U52102_at	NM_012932	1.28073	2.903181957
Rattus norvegicus transcribed sequences	**---**	rc_AI639142_at	---	1.27885	3.780313527
Olf-1/EBF associated Zn finger protein Roaz	**Roaz**	U92564_g_at	NM_053583	1.27619	3.940821135
glycine receptor, alpha 2 subunit	**Glra2**	X57281_at	NM_012568	1.27507	3.780313527
Rattus norvegicus transcribed sequences	**---**	rc_AI639521_at	---	1.27328	2.903181957
ribosome associated membrane protein 4	**RAMP4**	AF100470_at	NM_030835	1.27136	4.266201283
platelet-activating factor acetylhydrolase beta subunit (PAF-AH beta)	**Pafah1b1**	rc_AI234730_at	NM_031763	1.27109	3.940821135
proliferating cell nuclear antigen	**Pcna**	M24604_at	NM_022381	1.26833	3.780313527
calcitonin receptor	**Calcr**	L13040_s_at	NM_053816	1.26802	3.780313527
hairy and enhancer of split 1 (Drosophila)	**Hes1**	D13417_g_at	NM_024360	1.26743	4.876302628
pancreatic lipase	**Pnlip**	D88534_s_at	NM_013161	1.26675	3.780313527
Rattus norvegicus transcribed sequences	**---**	rc_AA799964_at	---	1.26464	3.780313527
cytochrome P450, IVA1	**Cyp4a1**	M14972_i_at	NM_175837	1.26340	4.876302628
carnitine palmitoyltransferase 1, liver	**Cpt1a**	L07736_at	NM_031559	1.26231	4.876302628
brain acidic membrane protein	**Basp1**	D14441_at	NM_022300	1.26001	3.780313527
S-100 related protein, clone 42C	**S100a10**	J03627_at	NM_031114	1.25712	4.266201283
Rattus norvegicus transcribed sequences	**---**	rc_AA893743_g_at	---	1.25544	3.780313527
cysteine rich protein 2	**Csrp2**	U44948_at	NM_177425	1.25498	4.266201283
---	**---**	X07944exon#1-12_s_at	---	1.25495	2.903181957
cyclin L	**Ccnl**	AF030091UTR#1_g_at	NM_053662	1.25233	4.876302628
solute carrier family 11 member 2	**Slc11a2**	AF008439_at	NM_013173	1.25224	2.903181957
acyl-coenzyme A:cholesterol acyltransferase	**Soat1**	D86373_s_at	NM_031118	1.25218	3.940821135
glutamate receptor, ionotropic, N-methyl-D-aspartate 3A	**Grin3a**	AF061945_g_at	---	1.24936	3.780313527
cerebroglycan	**Gpc2**	L20468_at	NM_138511	1.24907	4.266201283
topoisomerase II beta	**Top2b**	D14046_at	---	1.24804	4.876302628
chondroitin sulfate proteoglycan 3	**Cspg3**	AF060879_s_at	NM_031653	1.24717	4.266201283
cellular retinoic acid binding protein I	**Crabp1**	rc_AA875025_at	---	1.24457	4.876302628
adrenomedullin	**Adm**	D15069_s_at	NM_012715	1.24032	4.876302628
Bcl2-associated X protein	**Bax**	U59184_at	NM_017059	1.23951	4.876302628
Rattus norvegicus transcribed sequence with moderate similarity to protein ref:NP_002485.1 (H.sapiens) NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1 (6kD, KFYI) [Homo sapiens]	**---**	rc_AI104679_s_at	---	1.23711	4.266201283
Rattus norvegicus transcribed sequences	**---**	rc_AA859536_at	---	1.23592	3.940821135
Rattus norvegicus transcribed sequences	**---**	rc_AA860029_at	---	1.23586	3.940821135
cytochrome P450 2c22	**Cyp2c22**	M58041_s_at	NM_138512	1.23401	4.876302628
Rattus norvegicus transcribed sequence with strong similarity to protein sp:P00722 (E. coli) BGAL_ECOLI Beta-galactosidase (Lactase)	**---**	rc_AA859994_at	---	1.23342	4.876302628
Chorionic somatomammotropin hormone 1 variant; Placental lactogen-1	**Csh1v**	rc_AI179075_i_at	NM_033233	1.23287	4.266201283
cyclin B1	**Ccnb1**	X64589_at	NM_171991	1.23239	3.940821135
Rattus norvegicus similar to dimethylarginine dimethylaminohydrolase 1; NG,NG dimethylarginine dimethylaminohydrolase (LOC365956), mRNA	**---**	D86041_at	---	1.23180	4.876302628
fatty acid binding protein 5, epidermal	**Fabp5**	S69874_s_at	NM_145878	1.22974	3.940821135
variable coding sequence A1	**Vcsa1**	A07543cds_s_at	NM_012684	1.22931	4.266201283
protease (prosome, macropain) 28 subunit, alpha	**Psme1**	D45249_g_at	NM_017264	1.22924	4.266201283
---	**---**	X77117exon#1-3_at	---	1.22895	4.876302628

cyclin D1	**Ccnd1**	D14014_at	NM_171992	1.22667	2.435234865
microtubule-associated protein 6	**Mtap6**	AJ002556_s_at	NM_017204	1.22297	3.940821135
---	**---**	X57529cds_s_at	---	1.21046	4.876302628
ribosomal protein S4, X-linked	**Rps4x**	X14210cds_at	---	1.18362	3.940821135
			-		
DOWN-REGULATED GENES			-		
thyroid hormone responsive protein	**Thrsp**	K01934mRNA#2_at	NM_012703	0.39906	0.831810561
Rattus norvegicus Nclone10 mRNA	**---**	U31866_g_at	---	0.47015	0.831810561
Transferrin	**Tf**	D38380_g_at	NM_017055	0.49119	0.831810561
myelin and lymphocyte protein	**Mal**	U31367_at	NM_012798	0.51875	0.831810561
Transferrin	**Tf**	D38380_at	NM_017055	0.53227	0.831810561
kallikrein 6	**Klk6**	AF016269_at	NM_019175	0.53451	0.831810561
homer, neuronal immediate early gene, 1	**Homer1**	AB003726_at	NM_031707	0.53698	0.831810561
myelin-associated oligodendrocytic basic protein	**Mobp**	D28111_at	NM_012720	0.53918	0.831810561
apolipoprotein D	**Apod**	X55572_at	NM_012777	0.54264	0.831810561
Rat troponin-c mRNA	**---**	rc_AI639532_at	---	0.55172	0.831810561
---	**---**	rc_AA892551_f_at	---	0.57617	0.831810561
myelin-associated oligodendrocytic basic protein	**Mobp**	D28111_g_at	NM_012720	0.59637	0.831810561
---	**---**	rc_AA874877_r_at	---	0.60311	0.831810561
myelin oligodendrocyte glycoprotein	**Mog**	L21995_s_at	NM_022668	0.61224	0.831810561
crystallin, alpha B	**Cryab**	X60351cds_s_at	NM_012935	0.61292	0.831810561
myelin-associated oligodendrocytic basic protein	**Mobp**	D28110_g_at	NM_012720	0.61885	0.831810561
afadin	**Af6**	D90401_at	NM_013217	0.63107	1.370947035
Rattus norvegicus retroviral-like ovarian specific transcript 30-1 mRNA	**---**	U48828_g_at	---	0.63803	1.370947035
apolipoprotein C-I	**Apoc1**	X15512_at	NM_012824	0.64339	0.831810561
carbonic anhydrase 2	**Ca2**	X58294_at	NM_019291	0.64519	0.831810561
RAS guanyl releasing protein 1	**Rasgrp1**	AF081196_at	NM_019211	0.64721	1.370947035
stathmin-like 4	**Stmn4**	AF026529_s_at	---	0.64929	0.831810561
---	**---**	rc_AA799594_at	---	0.65539	2.008596819
---	**---**	rc_AI102044_at	---	0.65630	0.831810561
ring finger protein 28	**Rnf28**	rc_AI639465_r_at	NM_080903	0.65888	0.831810561
lysozyme	**Lyz**	rc_AA892775_at	NM_012771	0.65940	1.370947035
myelin oligodendrocyte glycoprotein	**Mog**	M99485_at	NM_022668	0.66050	0.831810561
---	**---**	X70141_r_at	---	0.66242	0.831810561
olfactory receptor	**LOC309574**	AF091563_r_at	---	0.66307	0.831810561
carbonic anhydrase 2	**Ca2**	U60578cds_s_at	NM_019291	0.66321	0.831810561
glutathione S-transferase, mu 1	**Gstm1**	H32189_s_at	NM_017014	0.66508	0.831810561
afadin	**Af6**	D90401_g_at	NM_013217	0.66667	0.831810561
cathepsin S	**Ctss**	L03201_at	NM_017320	0.66680	0.831810561
Rattus norvegicus transcribed sequences	**---**	rc_AI007824_g_at	---	0.66928	0.831810561
Rattus norvegicus transcribed sequences	**---**	rc_AA891690_g_at	---	0.67005	2.435234865
deoxycytidine kinase	**Dck**	L33894_at	NM_024158	0.67197	3.940821135
Rattus norvegicus similar to S-100 protein, alpha chain (LOC295214), mRNA	**---**	rc_AI228548_g_at	---	0.67279	0.831810561
Rat PMSG-induced ovarian mRNA, 3'sequence, N1	**---**	D84479_at	---	0.67470	3.780313527
---	**---**	AF028784cds#1_s_at	---	0.67531	0.831810561
purinergic receptor P2X, ligand-gated ion channel, 4	**P2rx4**	U47031_at	NM_031594	0.67637	0.831810561
MEGF1	**Fat2**	AB011527_at	NM_022954	0.67692	0.831810561

avian erythroblastosis oncogene B 3	**Erbb3**	U29339_at	NM_017218	0.68155	1.370947035
neurotensin receptor 2	**Ntsr2**	X97121_at	NM_022695	0.68834	0.831810561
cytochrome P450, family 27, subfamily a, polypeptide 1	**Cyp27**	M38566mRNA_s_at	NM_178847	0.69094	0.831810561
Rattus norvegicus transcribed sequences	**---**	rc_AI639170_at	---	0.69337	2.903181957
stearoyl-Coenzyme A desaturase 2	**Scd2**	M15114_at	NM_031841	0.69670	3.780313527
Rattus norvegicus transcribed sequences	**---**	rc_AA874805_at	---	0.69783	2.903181957
heat-responsive protein 12	**Hrsp12**	D49363_s_at	NM_031714	0.69849	1.370947035
ectonucleotide pyrophosphatase/phosphodiesterase 2	**Enpp2**	D28560_at	NM_057104	0.69910	0.831810561
crystallin, alpha B	**Cryab**	M55534mRNA_s_at	NM_012935	0.69912	0.831810561
Rattus norvegicus transcribed sequences	**---**	rc_AA891690_at	---	0.70103	2.903181957
secreted phosphoprotein 1	**Spp1**	M14656_at	NM_012881	0.70182	1.370947035
---	**---**	X83671cds_r_at	---	0.70462	0.831810561
myelin basic protein	**Mbp**	K00512_at	NM_017026	0.70604	0.831810561
---	**---**	K03045cds_s_at	---	0.70996	3.780313527
serum/glucocorticoid regulated kinase	**Sgk**	L01624_at	NM_019232	0.71101	2.435234865
Rattus norvegicus hypothetical gene supported by NM_053936 (LOC360403), mRNA	**---**	rc_AA848831_at	---	0.71152	0.831810561
cytochrome P450CMF1b	**Cyp2d5**	J02869mRNA_s_at	NM_173304	0.71157	0.831810561
Eph receptor A7	**Epha7**	U21954_at	NM_134331	0.71186	2.903181957
Rattus norvegicus transcribed sequence	**---**	rc_AI639471_r_at	---	0.71198	2.435234865
parvalbumin	**Pva**	rc_AI175539_at	NM_022499	0.71353	0.831810561
chemokine (C-X-C motif) ligand 2	**Cxcl2**	U45965_at	NM_053647	0.71378	4.876302628
---	**---**	L08495cds_s_at	---	0.71522	3.780313527
potassium voltage-gated channel, subfamily H (eag-related), member 1	**Kcnh1**	Z34264_at	NM_031742	0.71633	2.008596819
tropomodulin 1	**Tmod1**	U59241_at	NM_013044	0.71899	0.831810561
carbonic anhydrase 4	**Ca4**	S68245_g_at	NM_019174	0.71970	0.831810561
---	**---**	D37934_at	---	0.71982	2.008596819
Rattus norvegicus transcribed sequences	**---**	rc_AA866432_at	---	0.72186	0.831810561
---	**---**	rc_AA799671_at	---	0.72235	2.008596819
chimerin (chimaerin) 2	**Chn2**	rc_AI232194_at	NM_032084	0.72322	1.370947035
glycerol 3-phosphate dehydrogenase	**Gpd3**	AB002558_at	NM_022215	0.72603	0.831810561
proteasome (prosome, macropain) subunit, beta type 4	**Psmb4**	rc_AI172162_at	NM_031629	0.72621	0.831810561
Rattus norvegicus transcribed sequences	**---**	rc_AA800853_at	---	0.72883	3.780313527
---	**---**	X51531cds_g_at	---	0.72920	2.903181957
protein disulfide isomerase related protein (calcium-binding protein, intestinal-related)	**Erp70**	M86870_at	NM_053849	0.73317	1.370947035
fumarylacetoacetate hydrolase	**Fah**	M77694_at	NM_017181	0.73325	2.435234865
neuronal potassium channel alpha subunit	**Kv8.1**	X98564cds_at	NM_021697	0.73511	2.903181957
Rattus norvegicus transcribed sequence with strong similarity to protein sp:O43236 (H.sapiens) SEP4_HUMAN Septin 4 (Peanut-like protein 2) (Brain protein H5) (Cell division control-related protein 2) (hCDCREL-2) (Bradeion beta) (CE5B3 beta)	**---**	rc_AA800004_at	---	0.73664	0.831810561
solute carrier family 21 (organic anion transporter), member 5	**Slc21a5**	U88036_at	NM_131906	0.74072	1.370947035
---	**---**	U26356mRNA_s_at	---	0.74099	2.008596819
proteolipid protein	**Plp**	rc_AI072770_s_at	NM_030990	0.74110	0.831810561
cAMP responsive element modulator	**Crem**	S66024_at	NM_013086	0.74164	2.008596819

Rattus norvegicus transcribed sequence	---	rc_AI639098_at	---	0.74270	2.008596819
kangai 1	**Kai1**	rc_AI231213_g_at	NM_031797	0.74471	2.008596819
Mg87 protein	**Mg87**	AF095741_at	NM_134410	0.74517	4.266201283
aldehyde dehydrogenase family 1, member A1	**Aldh1a1**	AF001898_at	NM_022407	0.74614	2.903181957
myosin, heavy polypeptide 3	**Myh3**	K03467_s_at	NM_012604	0.74628	4.266201283
Rattus norvegicus transcribed sequence with moderate similarity to protein ref:NP_060369.1 (H.sapiens) hypothetical protein FLJ20607; tescalcin; likely ortholog of mouse tescalcin [Homo sapiens]	---	rc_AA892511_at	---	0.74700	1.370947035
retinoblastoma-like 2	**Rbl2**	rc_AI230602_at	NM_031094	0.74707	4.876302628
adenylyl cyclase-associated protein 2	**Cap2**	rc_AI145367_at	NM_053874	0.74797	0.831810561
Rattus norvegicus similar to kIAA0367 (LOC293823), mRNA	---	rc_AA894264_at	---	0.75082	3.780313527
Rattus norvegicus transcribed sequence with moderate similarity to protein sp:P07902 (H.sapiens) GAL7_HUMAN Galactose-1-phosphate uridylyltransferase	---	L05541_at	---	0.75139	1.370947035
potassium voltage gated channel, shaker related subfamily, beta member 1	**Kcnab1**	X70662_at	NM_017303	0.75175	2.435234865
---	---	M64733mRNA_s_at	---	0.75290	1.370947035
B-cell translocation gene 1	**Btg1**	L26268_at	NM_017258	0.75291	0.831810561
---	---	AF028784mRNA#1_s_at	---	0.75373	2.435234865
---	---	X62327cds_r_at	---	0.75497	2.008596819
Rattus norvegicus transcribed sequence with strong similarity to protein ref:NP_060904.1 (H.sapiens) goliath protein; likely ortholog of mouse g1-related zinc finger protein [Homo sapiens]	---	rc_AA891810_at	---	0.75520	2.435234865
Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	**Id3**	rc_AI171268_at	NM_013058	0.75603	4.876302628
FXYD domain-containing ion transport regulator 1	**Fxyd1**	rc_AA799645_g_at	NM_031648	0.75604	2.903181957
CD9 antigen	**Cd9**	X76489cds_at	---	0.75604	4.266201283
S100 protein, beta polypeptide	**S100b**	S53527mRNA_s_at	NM_013191	0.75796	4.266201283
gamma-aminobutyric acid A receptor, delta	**Gabrd**	L08496cds_s_at	NM_017289	0.75881	4.876302628
muscle glycogen phosphorylase	**Pygm**	L10669_g_at	---	0.75899	2.903181957
Cys2/His2 zinc finger protein (rKr1)	**rKr1**	U41164_at	NM_144757	0.76013	2.008596819
sterol-C4-methyl oxidase-like	**Sc4mol**	E12625cds_at	NM_080886	0.76023	2.903181957
liver mitochondrial glutaminase	**Ga**	J05499_at	NM_138904	0.76200	2.435234865
dopamine receptor 1A	**Drd1a**	S46131mRNA_r_at	NM_012546	0.76203	0.831810561
Mg87 protein	**Mg87**	AF095741_g_at	NM_134410	0.76213	3.780313527
potassium voltage gated channel, Shal-related family, member 3	**Kcnd3**	rc_AI230211_s_at	NM_031739	0.76244	4.266201283
Rattus norvegicus transcribed sequence with moderate similarity to protein pir:T50629 (H.sapiens) T50629 hypothetical protein DKFZp762L1710.1 - human (fragment)	---	rc_AA800258_at	---	0.76337	2.903181957
xanthine dehydrogenase	**Xdh**	rc_AI172247_at	NM_017154	0.76447	2.435234865
Rattus norvegicus transcribed sequences	---	rc_AA799534_at	---	0.76458	2.903181957
gamma-aminobutyric acid A receptor, delta	**Gabrd**	M35162_at	NM_017289	0.76509	2.903181957
peroxiredoxin 6	**Prdx6**	AF014009_at	NM_053576	0.76517	0.831810561
glycine methyltransferase	**Gnmt**	X06150cds_at	NM_017084	0.76603	3.940821135
calpain, small subunit 1	**Capns1**	U53859_at	---	0.76690	2.008596819

Rattus norvegicus transcribed sequence with strong similarity to protein ref:NP_060904.1 (H.sapiens) goliath protein; likely ortholog of mouse g1-related zinc finger protein [Homo sapiens]	---	rc_AA891810_g_at	---	0.76880	2.008596819
galectin-related inter-fiber protein	**Grifin**	AF082160_at	NM_057187	0.76896	3.780313527
Rattus norvegicus similar to glyoxylate reductase/hydroxypyruvate reductase (LOC298085), mRNA	---	rc_AA892799_s_at	---	0.76962	3.780313527
---	---	rc_AA859928_at	---	0.77063	2.903181957
D site albumin promoter binding protein	**Dbp**	J03179_at	NM_012543	0.77116	2.903181957
Rattus norvegicus transcribed sequence	---	rc_AI639471_f_at	---	0.77150	4.876302628
pyruvate dehydrogenase kinase 2	**Pdk2**	U10357_g_at	NM_030872	0.77193	3.780313527
proprotein convertase subtilisin/kexin type 4	**Pcsk4**	L14937cds#1_s_at	NM_133559	0.77248	2.903181957
Rattus norvegicus similar to APC-binding protein EB1 homolog (LOC300647), mRNA	---	rc_AA891727_at	---	0.77260	2.903181957
Rattus norvegicus transcribed sequences	---	rc_AA800549_at	---	0.77312	3.780313527
FXYD domain-containing ion transport regulator 1	**Fxyd1**	rc_AA799645_at	NM_031648	0.77347	2.903181957
glutathione S-transferase, mu 1	**Gstm1**	J02810mRNA_s_at	NM_017014	0.77369	2.435234865
cellular retinoic acid binding protein 2	**Crabp2**	U23407_at	NM_017244	0.77538	4.876302628
---	---	M90660exon_at	---	0.77647	4.266201283
lifeguard	**Lfg**	AF044201_at	NM_144756	0.77706	2.903181957
pregnancy up-regulated non-ubiquitously expressed CaM kinase	**Pnck**	D86556_i_at	NM_017275	0.77814	3.780313527
phosphatidylinositol 4-kinase	**Pik4cb**	rc_AI102103_g_at	NM_031083	0.77839	2.903181957
Rattus norvegicus similar to RING-finger protein MURF (LOC362708), mRNA	---	rc_AA800245_at	---	0.77950	2.903181957
Rattus norvegicus transcribed sequences	---	rc_AA894297_at	---	0.77963	3.780313527
Rab3B protein	**Rab3b**	AA799389_g_at	NM_031091	0.78252	2.903181957
complement component 3	**C3**	rc_AA894304_at	NM_016994	0.78284	4.266201283
proteolipid protein	**Plp**	rc_AI070277_s_at	NM_030990	0.78385	1.370947035
plasmolipin	**Z49858**	Z49858_at	NM_022533	0.78418	2.903181957
discs, large homolog 1 (Drosophila)	**Dlgh1**	rc_AI144926_s_at	NM_012788	0.78422	4.266201283
short stature homeobox 2	**Shox2**	AJ002259_g_at	NM_013028	0.78546	4.266201283
Rattus norvegicus Tclone4 mRNA	---	U30788_at	---	0.78569	4.876302628
peripheral myelin protein 22	**Pmp22**	S55427_s_at	NM_017037	0.78677	4.266201283
galanin	**Gal**	J03624_at	NM_033237	0.78824	3.780313527
---	---	U75927UTR#1_at	---	0.78992	2.435234865
Glucose-dependent insulinotropic peptide	**Gludins**	L08831_i_at	NM_019630	0.79211	4.266201283
guanidinoacetate methyltransferase	**Gamt**	J03588_at	NM_012793	0.79233	2.903181957
Rattus norvegicus transcribed sequences	---	rc_H31859_at	---	0.79395	4.266201283
gap junction membrane channel protein beta 1	**Gjb1**	X04070_at	NM_017251	0.79543	4.876302628
Rattus norvegicus transcribed sequences	---	rc_AA892541_at	---	0.79598	1.370947035
---	---	S87522_g_at	---	0.79600	4.266201283
calcium channel, voltage-dependent, alpha2/delta subunit 1	**Cacna2d1**	M86621_at	NM_012919	0.79639	3.780313527
sodium channel, voltage-gated, type 1, alpha polypeptide	**Scn1a**	M22253_at	NM_030875	0.79685	2.903181957
v-jun sarcoma virus 17 oncogene homolog (avian)	**Jun**	X17163cds_s_at	NM_021835	0.79742	2.903181957
proteolipid protein	**Plp**	M25888_at	NM_030990	0.79874	2.435234865
hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	**Hsd3b1**	rc_AI235576_s_at	NM_017265	0.80152	3.780313527
putative chloride channel (similar to Mm Clcn4-2)	**Clcn4-2**	Z36944cds_at	NM_022198	0.80158	2.008596819

CD9 antigen	**Cd9**	X76489cds_g_at	---	0.80167	3.780313527
3-hydroxy-3-methylglutaryl CoA lyase	**Hmgcl**	rc_AI171090_at	NM_024386	0.80230	4.266201283
cytochrome P450 monooxygenase	**Cyp2J3**	U40004_s_at	NM_175766	0.80317	4.266201283
myosin heavy chain, polypeptide 6	**Myh6**	rc_AI104924_f_at	NM_017239	0.80320	4.876302628
ubiquitin conjugating enzyme	**LOC81816**	rc_AA799612_at	NM_031138	0.80326	4.266201283
fibroblast growth factor 9	**Fgf9**	D14839_at	NM_012952	0.80525	4.876302628
stearoyl-Coenzyme A desaturase 2	**Scd2**	U67995_s_at	NM_031841	0.80532	3.940821135
CaM-kinase II inhibitor alpha	**LOC287005**	rc_AA858621_at	NM_173337	0.80569	4.876302628
cAMP responsive element modulator	**Crem**	U04835_at	NM_013086	0.80586	2.008596819
Max interacting protein 1	**Mxi1**	AF003008_at	NM_013160	0.80629	4.266201283
ephrin A1	**Efna1**	D38056_at	NM_053599	0.80672	4.266201283
pyridoxine 5-phosphate oxidase	**U91561**	rc_AA800211_at	NM_022601	0.80743	4.266201283
protease, serine, 8 (prostasin)	**Prss8**	rc_AA892468_g_at	NM_138836	0.80859	3.780313527
Rattus norvegicus transcribed sequences	**---**	rc_AA875362_at	---	0.80903	3.940821135
Rattus norvegicus cDNA clone MGC:72479 IMAGE:5600078, complete cds	**---**	rc_AA891877_at	---	0.80944	4.876302628
calpain, small subunit 1	**Capns1**	U53859_g_at	---	0.80973	4.876302628
glutamine synthetase 1	**Glns**	rc_AI232783_s_at	NM_017073	0.80992	4.266201283
Rattus norvegicus transcribed sequence with strong similarity to protein ref:NP_076424.1 (H.sapiens) chromosome 20 open reading frame 116 [Homo sapiens]	**---**	rc_AI638949_s_at	---	0.81126	4.876302628
Rattus norvegicus transcribed sequence with strong similarity to protein pir:T08783 (H.sapiens) T08783 hypothetical protein DKFZp586O0120.1 - human (fragment)	**---**	rc_AA892310_at	---	0.81420	2.903181957
solute carrier family 25 (mitochondrial carrier; dicarboxylate transporter), member 10	**Slc25a10**	rc_AA859666_i_at	NM_133418	0.81457	3.780313527
Rattus norvegicus transcribed sequence	**---**	rc_AA866259_at	---	0.81529	2.435234865
---	**---**	E12275cds_s_at	---	0.81713	4.876302628
protein tyrosine kinase 2 beta	**Ptk2b**	AF063890_s_at	NM_017318	0.81777	2.903181957
potassium channel erg3	**erg3**	AF016191_at	NM_131912	0.82728	3.780313527
secretory granule neuroendocrine protein 1	**Sgne1**	M63901_g_at	NM_013175	0.83321	4.876302628
interleukin 1 receptor accessory protein	**Il1rap**	U48592_g_at	NM_012968	0.84996	4.876302628

Table 4.4. mRNAs known to contain an iron responsive element. The table lists mRNAs known to contain an iron responsive element (IRE) and the function of the encoded proteins.

Table 4.4

mRNA containing IRE	Protein Function
H-Ferritin	Iron storage
L-Ferritin	Iron storage
Transferrin Receptor (TfR)	Iron uptake
Divalent metal transporter-1 (DMT-1)	Iron uptake
Ferroportin/IREG1/MTP1	Iron export
Aconitase	Tricarboxylic acid cycle
e-aminolevulinate synthase (eALAS)	Heme formation

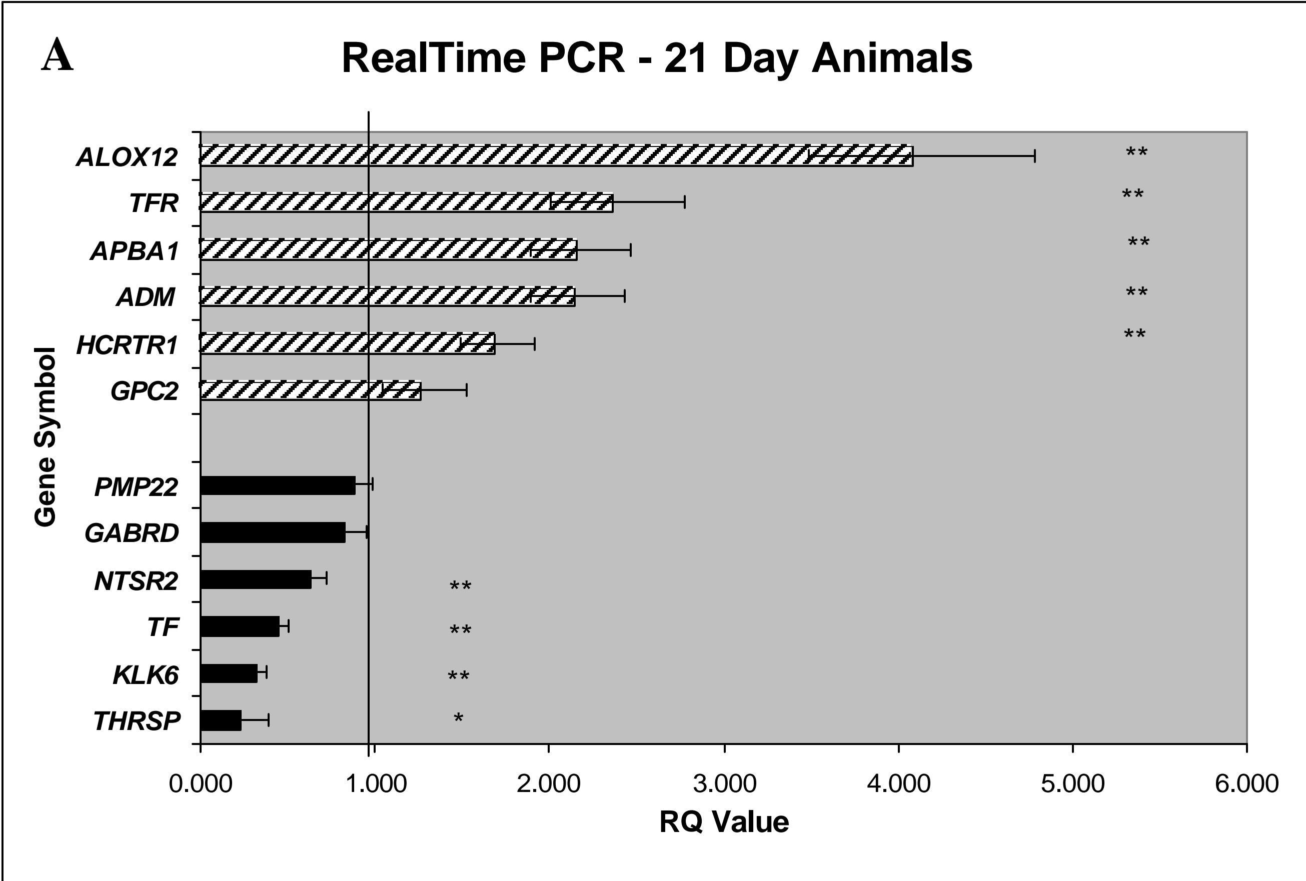
Figure 4.1. Gene clusters from 21-day old animals. Several significant gene clusters of interest were identified, including: myelin-related, signal transduction, channel/pore class transporter and alpha-type channel activity & ion channel activity, DNA binding, transitional metal binding, and solute carrier family members. This figure illustrates these clusters, the number of genes in each, and the direction of change.

21-Day Animals ~ Cluster Analysis

	Number of Genes	UP or DOWN Regulated
1. Myelin related	6	↓
2. Solute carrier family	8	2 ↓ 6 ↑
3. Signal transduction	13	↓
4. Channel/Pore class transporter & Ion channel activity	11	↓
5. DNA binding	8	↑
6. Transitional metal binding	3	↓

Figure 4.1

Figure 4.2. Real-time PCR verification of gene changes from animals both 21 days of age and six months of age. From the over 300 genes identified in the 21-day animals, twelve genes were selected for verification by real-time PCR using RNA from brains from the same animals studies in the microarray analysis. Four iron-deficient animals and four control animals were tested in each real-time PCR experiment. Of the twelve genes, six were up-regulated and six were down-regulated. The verification results are shown in, displayed as mean RQ value for each gene. (A). In the six month animals, a total of twelve genes were identified as significantly changed. Of the twelve genes found, all were down-regulated, and seven were either estimated sequence tags or transcribed sequences, leaving five genes for further investigation. The RQ value of the real-time PCR verification of all five genes are shown in (B). Error bars display the standard error of the mean (*p<0.05 and ** p<0.005).



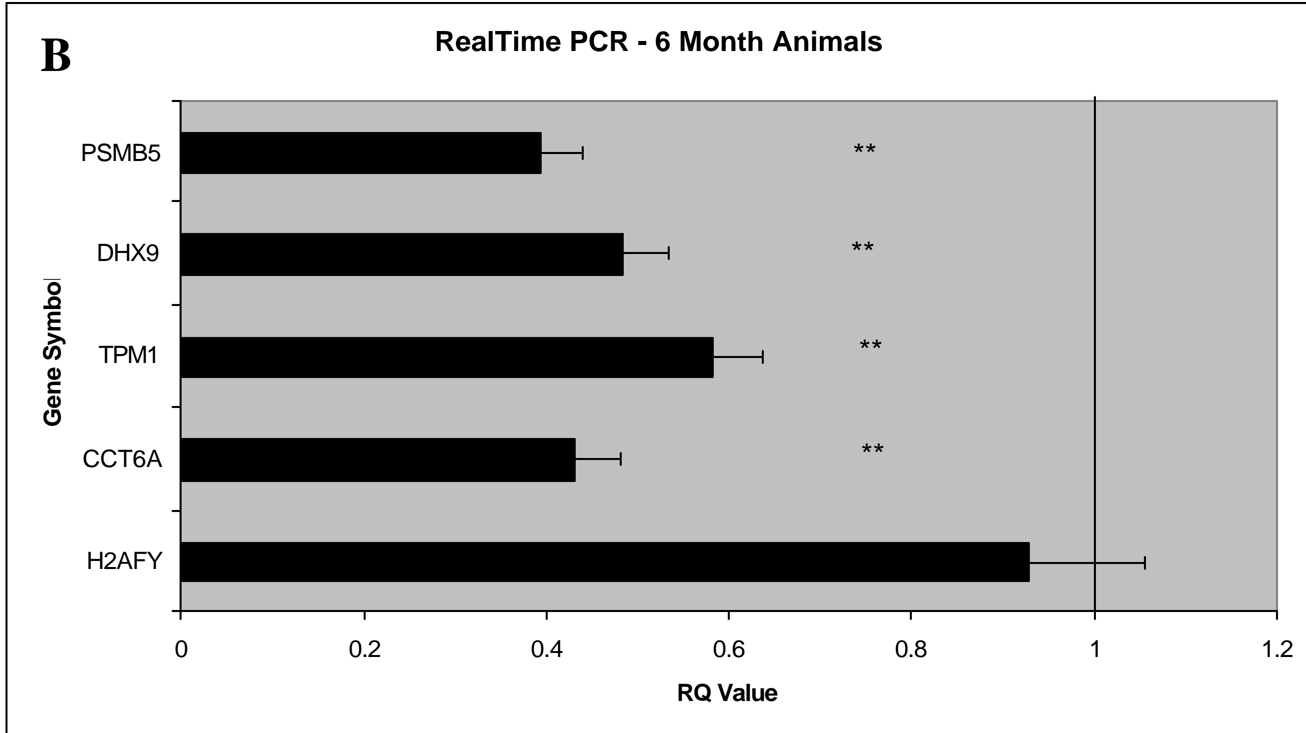


Figure 4.2

Figure 4.3. Real-time PCR verification of gene changes from biological replicates of 21-day-old animals. This figure displays the results of real-time PCR on biological replicates of the 21-day-old animals; brains from eight different animals (four control, four iron-deficient) were used to verify the change in each gene. The ID animals underwent the same 21-day iron-deficient diet as the animals examined in the microarray analysis. Six of the twelve genes were randomly selected for verification, of which four were up-regulated and two were down-regulated. The direction of change of the six genes is in agreement with the PCR verification for the brains of the original animals. Error bars display the standard error of the mean (*p<0.05 and ** p<0.005).

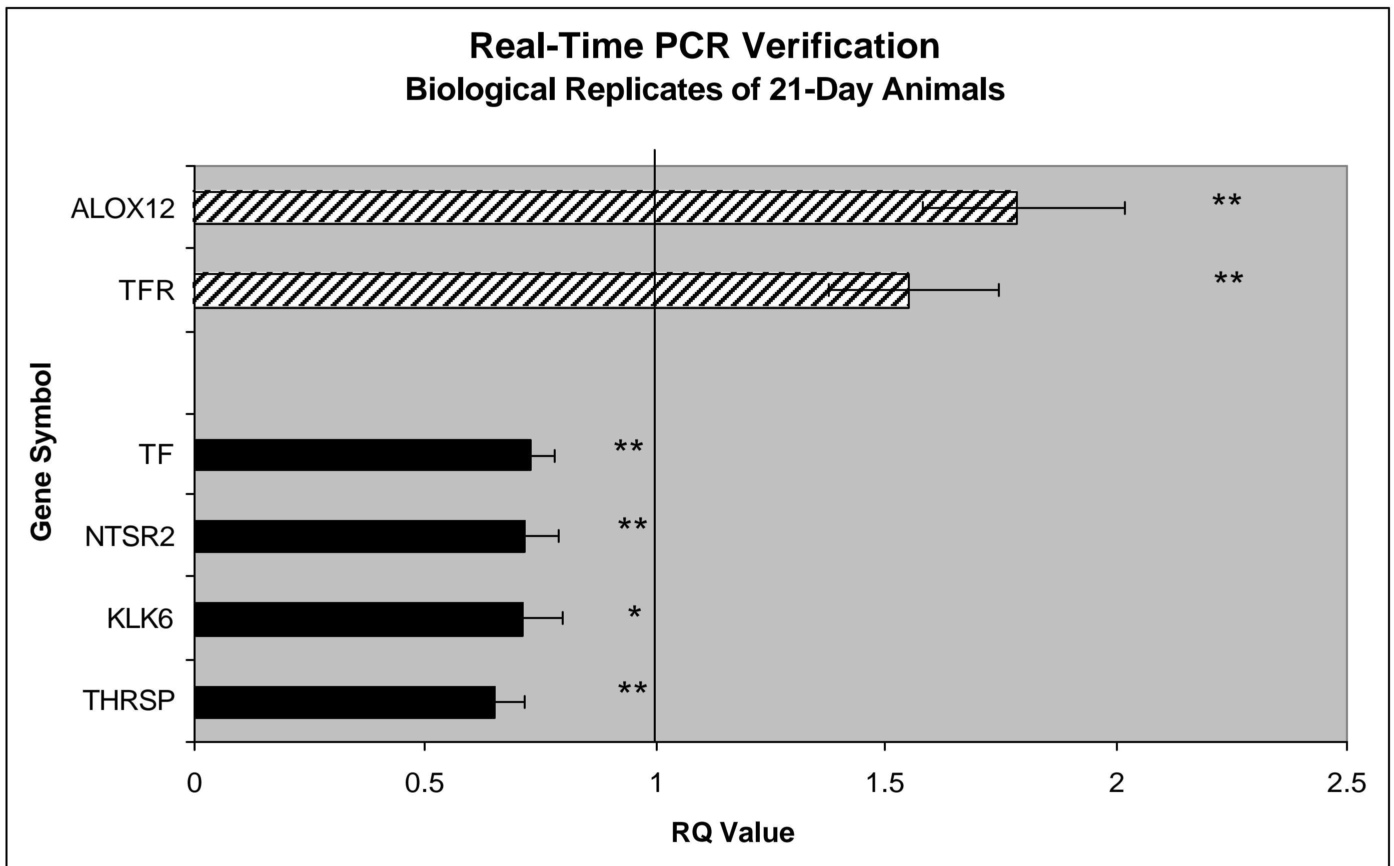


Figure 4.3

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Chapter 5

Iron Deficiency over the Lifetime

Future Directions and Final Conclusions

The studies in the preceding chapters were designed as diverse approaches to investigate the effects of brain iron deficiency. The first experiments were designed to investigate the levels of the iron-related proteins transferrin, whole-molecule ferritin, H-ferritin, and L-ferritin in Restless Legs Syndrome (RLS), with the goal of attaining a profile of these proteins in the cerebrospinal fluid (CSF) and understanding the cause of any changes in the levels of these proteins in samples from RLS patients compared to samples from controls. Previous reports have suggested a role for iron in the pathology of RLS (Allen, Barker et al. 2001; Connor, Boyer et al. 2003; Connor, Wang et al. 2004; Clardy, Earley et al. 2005; Earley, Allen et al. 2005; Mizuno, Mihara et al. 2005), but a large scale, comprehensive analysis was necessary to attain a definitive understanding of which iron-related proteins and peptides were altered in the CSF of RLS patients. Our findings have established a profile of these proteins in CSF that differentiates the two distinct RLS phenotypes, early- and late-onset RLS. Future studies of protein in the CSF of RLS patients would benefit from the utilization of an iTRAQ-type system to expand the current understanding of RLS profile at the protein level. Relative protein quantitation of CSF by iTRAQ could identify novel protein candidates not previously considered in the investigation of RLS pathology. Additionally, iTRAQ analysis would provide a more comprehensive protein profile, lending further support to the use of CSF as a diagnostic biomarker for RLS, and potentially further defining syndrome phenotypes. This would be particularly useful in RLS because the pathology appears to occur in the central nervous system, and therefore serum is not likely to be as useful as CSF in obtaining a diagnostic profile. Future studies of fluids from RLS

patients should also include both morning and evening sampling scenarios to elucidate any possible circadian effects, as possibly indicated by our CSF studies of transferrin.

Another objective of our investigations was to determine if the iron-signaling hormone hepcidin was present in the CSF, and if so, if its levels were altered in RLS. We provided evidence for hormonal iron signaling in the CNS by demonstrating the presence of hepcidin in the brain and significant differences in the amount of hepcidin in the brain and pro-hepcidin in the CSF in RLS patients compared to controls. Specifically, hepcidin was increased in brains from RLS patients compared to control brains, but pro-hepcidin was decreased in CSF from RLS patients compared to control CSF. It is possible that these differences reflect different roles of hepcidin, but it is also possible that the differences may underlie RLS pathology. While these data clearly showed that hepcidin is responsive to the iron-related changes in RLS, it remains to be determined if hepcidin is part of the problem in RLS, or if it is attempting to correct a brain iron imbalance in RLS. In general, future studies should attempt to further clarify the relationship between brain and CSF iron status in RLS. Investigation of other cell types and brain regions for the presence of both hepcidin protein and message (via immunocytochemistry and *in situ* hybridization) may aid in understanding the source and function of hepcidin in the brain.

The presence of hepcidin in RLS also highlights the need for the consideration of several proteins as yet not investigated in RLS, including ferroportin and ceruloplasmin. Several studies have clearly linked hepcidin to ferroportin, but few have considered the established association of ferroportin and ceruloplasmin (Cp) in interpreting the effects of hepcidin. Ceruloplasmin is expressed in the ependyma, the choroid plexus, and in astrocytes, particularly in perivascular astrocytes and those surrounding dopaminergic neurons in the substantia nigra. David and

coworkers have suggested that the glycosylphosphatidylinositol-anchored ceruloplasmin (GPI-Cp) expressed by astrocytes is the major form of Cp in the central nervous system, because serum Cp does not cross the blood-brain barrier (Patel and David 1997). They determined that FP is co-localized with GPI-Cp on the surface of cultured astrocytes, and that cultured astrocytes from Cp -/- knockout mice demonstrated significantly impaired iron efflux (Jeong and David 2003). Jeong et al suggest that it is possible that the inability of FP to act without Cp is intended to be a protective mechanism acutely, but that a chronic lack of Cp results in intracellular iron accumulation that overwhelms the cell. The role of Cp in iron overload may provide clues to the converse situation that is possibly reflected in RLS and iron deficiency, making it important to consider of the role of Cp in all future studies of FP-hepcidin interactions in the central nervous system. For example, in the context of our proposed model of hepcidin action in the neuromelanin cells of brain, over-expression of Cp could allow increased iron uptake locally into specific individual cells but worsen the total brain iron deficiency.

Our third objective in these studies was to understand both the acute and the long-term consequences of developmental iron deficiency on the brain. We utilized microarray analysis of the brain of the developmental iron-deficient rat model, and studied rats born to iron-deficient mother at two different ages: at 21 days, while weaning and iron-deficient; and at six months, after a recovery period consisting of normal iron intake for five months. Over 300 genes were significantly changed in the 21-day-old animals, compared to five significantly changed genes in the six-month-old animals. The data suggest that the gene array profiles obtained at postnatal day 21 reflect a brain under development in a metabolically compromised setting, and given adequate and well-timed intervention, the changes seem to be mostly reversible. In the six month animals, the significantly changed genes reflect changes that are long-term and that could

underlie the neurological deficits reported for iron deficiency. Further experiments could utilize additional animal groups with longer duration iron-deficiency to precisely determine the length of iron deficiency after which the changes become irreversible. Future studies should be designed to interrogate the specific protein products of the identified genes. Specifically, studies of message distribution in brain using *in situ* hybridization, complemented by immunohistochemical studies of protein distribution, would clarify the specific brain regions affected in the up- or down-regulation of a given gene in ID. The results of this study would establish if the observed gene changes translate into changes in protein expression levels, and determine which specific areas of the brain are most affected by iron deficiency for each specific gene/protein. Accordingly, changes in proteins specific to brain regions could help to localize the known behavioral and motor abnormalities that result from developmental iron deficiency to specific functional protein pathways.

Because the changes from the six-month-old animals reflect long term consequences of an early iron deficiency, in the final experiments of the thesis we tested the hypothesis that these genes changed in the six-month-old animals may be involved in the iron-related pathology of RLS. Specifically, we hypothesized that although RLS is primarily a condition that manifests in adults, it may be related to brain iron deficiency or iron misregulation earlier in development. Accordingly, we performed real time PCR on caudate nuclei from three brains from RLS patients and three control brains for the five genes that showed changes in the six-month-old animals by microarray analysis. We failed to find any significant differences in the mRNA expression of these genes in RLS vs. control, as shown in Figure 5.1. These findings raise several possibilities. The first is that human caudate tissue may not be an appropriate tissue to test for changes that were global in rat brain. Alternatively, the long-term effects observed in the

six-month-old animals may occur only as a result of brain iron deficiency in early development. If the latter is the case, this could be important in arguing that RLS reflects a state of acute iron misregulation, rather than a condition resulting from the long-term effects on the brain of developmental iron deficiency. Several lines of evidence suggest a state of acute iron misregulation in RLS, including the rapid response of RLS patients to intravenous iron supplementation (Earley, Heckler et al. 2004; Earley, Heckler et al. 2005), as well as the rapid development of secondary RLS in conditions of compromised iron, such as pregnancy and renal failure (Allen 2004). For this reason, future investigation should focus on candidate genes from the 21-day old animals, as these animals are a model of an acutely iron-deficient state. The genes that are changed in this model reflect the adaptive responses of the brain to inadequate levels of iron. In the case of the animal, the inadequate levels were due to a systemic iron deficiency. In RLS, the brain iron abnormalities likely have a different cause, such as defective iron transport, uptake, or regulation in the brain. The consequence of the defect in RLS, however, possibly shares the same end result as iron deficiency: there is a lack of usable iron in the brain at the cellular level.

Several genes changed in the 21-day-old animals appear to support the proposed similarities between RLS and the brain effects of iron deficiency. For example, one of these up-regulated genes, hypocretin receptor 1, could be involved in the altered hypocretin transmission believed to be involved in RLS. An increase in CSF hypocretin-1 levels was observed in patients with early-onset RLS (Allen, Mignot et al. 2002). Another gene, neurotensin receptor 2, which was down-regulated, recognizes neurotensin. Neurotensin is a modulator of dopaminergic transmission. Dopamine levels are directly involved in RLS, as indicated by the effectiveness of dopamine agonists in treating RLS symptoms (Hening, Allen et al. 2004; Ondo, Romanyshyn et

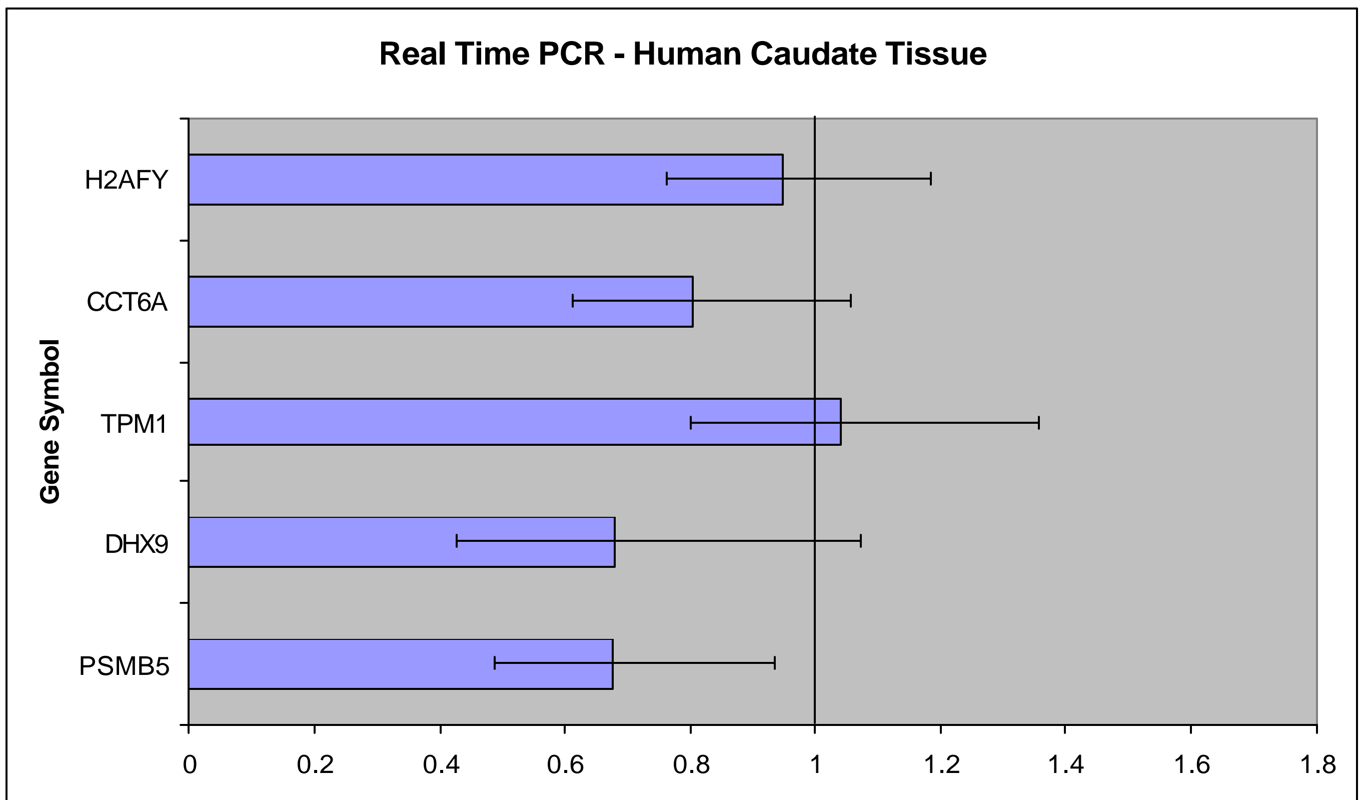
al. 2004), as well as the data demonstrating altered dopamine receptor levels in RLS brain (unpublished data). In addition to the real time PCR studies based on the developmental iron-deficient rat, a specific microarray analysis of brains from RLS patients could extend the current protein findings to the gene level, or, alternatively, demonstrate that most iron management abnormalities in RLS occur at the post-transcriptional level. The microarray study would also be a useful method to screen the growing list of candidate genes in RLS arising from linkage studies, given that such candidate genes are based largely on chromosomal location, without a specifically proposed functional relationship to the pathology.

In general, studies of iron deficiency in humans in the future should define the life-long conditions associated with iron deficiency at all times during the life span. Iron-deficient children must be followed out beyond adolescence, and studies should be done to determine those diseases that iron-deficient individuals have a greater risk of acquiring – or, perhaps equally as important – those diseases that iron-deficiency may protect against. For example, the association between secondary RLS and neurological conditions, such as Charcot-Marie-Tooth type 2 disease, Parkinson's disease, and spinocerebellar ataxia, presents an excellent opportunity for investigation. If RLS is more prevalent in patients with Parkinson's disease than it is in the general population, as suggested by Ondo and colleagues (Ondo, Vuong et al. 2002), it might be important to screen for shared (and different) iron or dopamine abnormalities in the CSF or brain tissue of patients with both conditions. In Charcot-Marie-Tooth type 2 disease, similar screening may also be useful to establish a possible common iron or dopamine abnormality. Additionally, gene and protein studies could ascertain differences between type 2 and the other forms of Charcot-Marie-Tooth diseases to determine why the co-morbidity is specific to the type 2 form of the disease.

In summary, our findings have had a significant impact on the current understanding of the relationship between the central nervous system and iron deficiency. We established a distinct CSF profile for RLS. We provided novel information regarding the presence of hepcidin in the CNS, and we found significant alterations in hepcidin levels in RLS. In the developmental iron-deficient rat brain, we identified numerous adaptive and compensatory changes during iron deficiency, as well as several long term consequences, providing necessary information in elucidating the biological basis of the neurological and behavioral alterations resulting from iron deficiency.

Figure 5.1. Real-time PCR of RLS and control caudate. This figure displays the results of real-time PCR on caudate nuclei from patients with RLS and controls. Caudate from six different brains (three controls, three RLS) was utilized for investigation of the mRNA expression of the five genes shown below. Values are normalized to a control value of one. None of the changes were statistically significant ($p > 0.1$). Error bars display the standard error of the mean.

H2AFY: H2A histone family, member Y; CCT6A: Chaperonin containing TCP1, subunit 6A (zeta 1); TPM1: Tropomyosin 1 (alpha); DHX9: DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 9 (RNA helicase A, nuclear DNA helicase II; leukophysin); PSMB5: Proteasome (prosome, macropain) subunit, beta type 5.



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Appendix
Section 1

H-ferritin Immunoblot Results

H file name	H-ferritin (ng)	H-frtn protein-Normalized	EarlyLate Dx	Dx	Sample #	H concentr
H110S 28May	0.45		S	standard	H Standard	0.5
H110S 28May	0.72		S	standard	H Standard	1
H110S 28May	1.59		S	standard	H Standard	1.5
H110S 28May	2.13		S	standard	H Standard	2
H110S 28May	3.95		S	standard	H Standard	4
H110S 28May	4.88		S	standard	H Standard	7
H110S 28May	5.16		S	standard	H Standard	10
H110S 28May	0.53	0.68	C	Control	5616	10
H110S 28May	1.23	1.55	C	Control	5616	20
H110S 28May	1.63	2.1	C	Control	6712	10
H110S 28May	3.08	3.88	C	Control	6712	20
H110S 28May	0.52	0.67	E	RLS	2791	10
H110S 28May	1.08	1.36	E	RLS	2791	20
H110S 28May	0.95	1.23	L	RLS	5590	10
H110S 28May	2.24	2.83	L	RLS	5590	20
H110S 28May	4.81	6.2	C	Control	7312	10
H110S 28May	3.05	3.85	C	Control	7312	20
H110S 28May	0.67	0.86	L	RLS	6975	10
H110S 28May	1.47	1.85	L	RLS	6975	20
H110S 28May	0.79	1.02	L	RLS	74213	10
H110S 28May	1.63	2.06	L	RLS	74213	20
H110S 28May	1.07	1.38	P1	H110S	Pooled	10
H110S 28May	2.22	2.8	P2	H110s	Pooled	20
H230S 28May	0.47		S	standard	H Standard	0.5
H230S 28May	1.01		S	standard	H Standard	1
H230S 28May	1.68		S	standard	H Standard	1.5
H230S 28May	1.84		S	standard	H Standard	2
H230S 28May	2.17		S	standard	H Standard	4
H230S 28May	2.37		S	standard	H Standard	7
H230S 28May	2.42		S	standard	H Standard	10
H230S 28May	0.83	1.32	C	Control	7326	10
H230S 28May	1.82	3.29	C	Control	7326	20
H230S 28May	0.56	0.89	E	RLS	6959	10
H230S 28May	1.25	2.26	E	RLS	6959	20
H230S 28May	0.8	1.27	L	RLS	52303	10
H230S 28May	1.49	2.69	L	RLS	52303	20
H230S 28May	2.07	3.28	C	Control	7356	10
H230S 28May	1.95	3.52	C	Control	7356	20
H230S 28May	0.32	0.51	E	RLS	6956	10
H230S 28May	0.79	1.43	E	RLS	6956	20
H230S 28May	0.25	0.4	L	RLS	6465	10
H230S 28May	0.55	0.99	L	RLS	6465	20
H230S 28May	1.09	1.73	E	RLS	4244	10
H230S 28May	1.72	3.11	E	RLS	4244	20
H230S 28May	0.87	1.38	P1	H230S	Pooled	10
H230S 28May	1.55	2.8	P2	H230s	Pooled	20

H110S 18May	0.38		S	standard	H Standard	0.5
H110S 18May	0.77		S	standard	H Standard	1
H110S 18May	2.04		S	standard	H Standard	2
H110S 18May	4.41		S	standard	H Standard	4
H110S 18May	6.75		S	standard	H Standard	7
H110S 18May	6.88		S	standard	H Standard	10
H110S 18May	1.2	0.78	E	RLS	12698	10
H110S 18May	3.31	2.01	E	RLS	12698	20
H110S 18May	0.8	0.52	L	RLS	3443	10
H110S 18May	2.1	1.28	L	RLS	3443	20
H110S 18May	0.42	0.27	C	Control	4461	10
H110S 18May	0.82	0.5	C	Control	4461	20
H110S 18May	0.9	0.59	L	RLS	5055	10
H110S 18May	2.86	1.74	L	RLS	5055	20
H110S 18May	1.41	0.92	E	RLS	12380	10
H110S 18May	3.52	2.14	E	RLS	12380	20
H110S 18May	2.12	1.38	C	Control	4808	10
H110S 18May	4.88	2.97	C	Control	4808	20
H110S 18May	2.1	1.37	L	RLS	5384	10
H110S 18May	4.83	2.94	L	RLS	5384	20
H110S 18May	2.12	1.38	P1	H110S	Pooled	10
H110S 18May	4.6	2.8	P2	H110S	Pooled	20
H210S 18May	0.59		S	standard	H Standard	0.5
H210S 18May	0.91		S	standard	H Standard	1
H210S 18May	1.99		S	standard	H Standard	2
H210S 18May	4.01		S	standard	H Standard	4
H210S 18May	5		S	standard	H Standard	7
H210S 18May	5.43		S	standard	H Standard	10
H210S 18May	2.41	1.58	C	Control	71861	10
H210S 18May	4.14	2.75	C	Control	71861	20
H210S 18May	4.38	2.86	C	Control	2623	10
H210S 18May	5.42	3.6	C	Control	2623	20
H210S 18May	1.07	0.7	E	RLS	9	10
H210S 18May	2.57	1.71	E	RLS	9	20
H210S 18May	1.85	1.21	C	Control	4923	10
H210S 18May	3.88	2.58	C	Control	4923	20
H210S 18May	1.49	0.97	L	RLS	5000	10
H210S 18May	3.36	2.23	L	RLS	5000	20
H210S 18May	0.79	0.52	E	RLS	7495	10
H210S 18May	2.03	1.35	E	RLS	7495	20
H210S 18May	0.75	0.49	C	Control	1931	10
H210S 18May	1.89	1.26	C	Control	1931	20
H210S 18May	2.11	1.38	P1	L230S	Pooled	10
H210S 18May	4.21	2.8	P2	L230S	Pooled	20

H110S 12May	0.34		S	standard	H Standard	0.5
H110S 12May	1.07		S	standard	H Standard	1
H110S 12May	1.98		S	standard	H Standard	2
H110S 12May	2.45		S	standard	H Standard	4
H110S 12May	2.88		S	standard	H Standard	7
H110S 12May	3.1		S	standard	H Standard	10
H110S 12May	0.11		S	standard	H Standard	0
H110S 12May	0.66	0.91	C	Control	3	10
H110S 12May	1.69	2.22	C	Control	3	20
H110S 12May	0.43	0.59	E	RLS	4	10
H110S 12May	1.02	1.34	E	RLS	4	20
H110S 12May	1.65	2.28	L	RLS	4743	10
H110S 12May	2.68	3.52	L	RLS	4743	20
H110S 12May	0.66	0.91	C	Control	7	10
H110S 12May	1.7	2.23	C	Control	7	20
H110S 12May	0.88	1.21	E	RLS	40047	10
H110S 12May	1.87	2.46	E	RLS	40047	20
H110S 12May	1.33	1.84	L day	RLS	2	10
H110S 12May	2.42	3.18	L day	RLS	2	20
H110S 12May	1	1.38	P1	P1	Pooled	10
H110S 12May	2.13	2.8	P2	P2	Pooled	20

H210S 12May	0.38		S	standard	H Standard	0.5
H210S 12May	1.13		S	standard	H Standard	1
H210S 12May	1.95		S	standard	H Standard	2
H210S 12May	2.89		S	standard	H Standard	4
H210S 12May	3.28		S	standard	H Standard	7
H210S 12May	3.57		S	standard	H Standard	10
H210S 12May	0.04		S	standard	H Standard	0
H210S 12May	0.28	0.35	C	Control	1931	10
H210S 12May	0.75	1.01	C	Control	1931	20
H210S 12May	0.31	0.39	E	RLS	1637	10
H210S 12May	0.81	1.09	E	RLS	1637	20
H210S 12May	0.2	0.25	L	RLS	1162	10
H210S 12May	0.54	0.73	L	RLS	1162	20
H210S 12May	1.43	1.81	L	RLS	4954	10
H210S 12May	2.46	3.31	L	RLS	4954	20
H210S 12May	0.36	0.46	E	RLS	8	10
H210S 12May	0.9	1.21	E	RLS	8	20
H210S 12May	0.33	0.42	C	Control	3631	10
H210S 12May	1.12	1.51	C	Control	3631	20
H210S 12May	1.09	1.38	P1	P1	Pooled	10
H210S 12May	2.08	2.8	P2	P2	Pooled	20

L-ferritin Immunoblot Results

L file name	L-ferritin (ng)	L-frtn protein-Normalized	Dx	Sample #	L concentr
L1 28May04	0.35		standard	H Standard	0.5
L1 28May04	0.84		standard	H Standard	1
L1 28May04	1.73		standard	H Standard	1.5
L1 28May04	2.32		standard	H Standard	2
L1 28May04	3.74		standard	H Standard	4
L1 28May04	4.32		standard	H Standard	7
L1 28May04	4.55		standard	H Standard	10
L1 28May04	0.68	0.76	Control	5616	10
L1 28May04	2.02	2.71	Control	5616	20
L1 28May04	2.45	2.73	Control	6712	10
L1 28May04	3.78	5.07	Control	6712	20
L1 28May04	0.48	0.54	RLS	2791	10
L1 28May04	1.35	1.81	RLS	2791	20
L1 28May04	1.01	1.13	RLS	5590	10
L1 28May04	2.61	3.5	RLS	5590	20
L1 28May04	4.45	4.96	Control	7312	10
L1 28May04	3.56	4.77	Control	7312	20
L1 28May04	0.54	0.6	RLS	6975	10
L1 28May04	1.6	2.15	RLS	6975	20
L1 28May04	0.61	0.68	RLS	74213	10
L1 28May04	1.6	2.15	RLS	74213	20
L1 28May04	1.04	1.16	H110S	Pooled	10
L1 28May04	2.26	3.03	H110s	Pooled	20
L230S 28may	0.3		standard	H Standard	0.5
L230S 28may	0.99		standard	H Standard	1
L230S 28may	1.56		standard	H Standard	1.5
L230S 28may	2.48		standard	H Standard	2
L230S 28may	3.66		standard	H Standard	4
L230S 28may	4.32		standard	H Standard	7
L230S 28may	4.5		standard	H Standard	10
L230S 28may	1.02	1.29	Control	7326	10
L230S 28may	3.1	3.79	Control	7326	20
L230S 28may	0.58	0.73	RLS	6959	10
L230S 28may	1.63	1.99	RLS	6959	20
L230S 28may	0.9	1.13	RLS	52303	10
L230S 28may	2.22	2.71	RLS	52303	20
L230S 28may	3.39	4.27	Control	7356	10
L230S 28may	3.36	4.11	Control	7356	20
L230S 28may	0.26	0.33	RLS	6956	10
L230S 28may	0.98	1.2	RLS	6956	20
L230S 28may	0.13	0.16	RLS	6465	10
L230S 28may	0.58	0.71	RLS	6465	20
L230S 28may	1.39	1.75	RLS	4244	10
L230S 28may	2.96	3.62	RLS	4244	20
L230S 28may	0.92	1.16	H230S	Pooled	10
L230S 28may	2.48	3.03	H230s	Pooled	20

L130S 11Jun	0.49		standard	H Standard	0.5
L130S 11Jun	1.16		standard	H Standard	1
L130S 11Jun	1.36		standard	H Standard	1.5
L130S 11Jun	1.43		standard	H Standard	2
L130S 11Jun	1.6		standard	H Standard	4
L130S 11Jun	1.62		standard	H Standard	7
L130S 11Jun	1.7		standard	H Standard	10
L130S 11Jun	0.22	0.65	RLS	12698	10
L130S 11Jun	0.59	2.01	RLS	12698	20
L130S 11Jun	0.1	0.3	RLS	3443	10
L130S 11Jun	0.34	1.16	RLS	3443	20
L130S 11Jun	0.85	2.53	Control	4461	10
L130S 11Jun	1.11	3.78	Control	4461	20
L130S 11Jun	0.22	0.65	RLS	5055	10
L130S 11Jun	0.55	1.87	RLS	5055	20
L130S 11Jun	0.21	0.62	RLS	12380	10
L130S 11Jun	0.65	2.21	RLS	12380	20
L130S 11Jun	0.32	0.95	Control	4808	10
L130S 11Jun	0.94	3.2	Control	4808	20
L130S 11Jun	0.45	1.34	RLS	5384	10
L130S 11Jun	0.98	3.34	RLS	5384	20
L130S 11Jun	0.39	1.16	H110S	Pooled	10
L130S 11Jun	0.89	3.03	H110S	Pooled	20
L230S 11Jun	0.69		standard	H Standard	0.5
L230S 11Jun	1.22		standard	H Standard	1
L230S 11Jun	1.56		standard	H Standard	1.5
L230S 11Jun	1.69		standard	H Standard	2
L230S 11Jun	1.99		standard	H Standard	4
L230S 11Jun	2.04		standard	H Standard	7
L230S 11Jun	2.19		standard	H Standard	10
L230S 11Jun	0.61	2.08	Control	71861	10
L230S 11Jun	1.23	3.55	Control	71861	20
L230S 11Jun	1.25	4.26	Control	2623	10
L230S 11Jun	1.74	5.02	Control	2623	20
L230S 11Jun	0.09	0.31	RLS	9	10
L230S 11Jun	0.56	1.62	RLS	9	20
L230S 11Jun	0.17	0.58	Control	4923	10
L230S 11Jun	0.96	2.77	Control	4923	20
L230S 11Jun	0.22	0.75	RLS	5000	10
L230S 11Jun	0.67	1.93	RLS	5000	20
L230S 11Jun	0.14	0.48	RLS	7495	10
L230S 11Jun	0.3	0.87	RLS	7495	20
L230S 11Jun	0.02	0.07	Control	1931	10
L230S 11Jun	0.27	0.78	Control	1931	20
L230S 11Jun	0.34	1.16	L230S	Pooled	10
L230S 11Jun	1.05	3.03	L230S	Pooled	20

L110S 12May	0.26		standard	H Standard	0.5
L110S 12May	0.6		standard	H Standard	1
L110S 12May	1.93		standard	H Standard	2
L110S 12May	5		standard	H Standard	4
L110S 12May	7.65		standard	H Standard	7
L110S 12May	8.93		standard	H Standard	10
L110S 12May	0.13		standard	H Standard	0
L110S 12May	2.13	0.94	Control	3	10
L110S 12May	5.76	2.5	Control	3	20
L110S 12May	0.91	0.4	RLS	4	10
L110S 12May	2.94	1.28	RLS	4	20
L110S 12May	5.14	2.26	RLS	4743	10
L110S 12May	9.69	4.21	RLS	4743	20
L110S 12May	1.86	0.82	Control	7	10
L110S 12May	5.09	2.21	Control	7	20
L110S 12May	2.24	0.98	RLS	40047	10
L110S 12May	5.88	2.55	RLS	40047	20
L110S 12May	3.16	1.39	RLS	2	10
L110S 12May	6.91	3	RLS	2	20
L110S 12May	2.64	1.16	P1	Pooled	10
L110S 12May	6.98	3.03	P2	Pooled	20

L210S 12May	0.23		standard	H Standard	0.5
L210S 12May	0.56		standard	H Standard	1
L210S 12May	2.21		standard	H Standard	2
L210S 12May	4.65		standard	H Standard	4
L210S 12May	7.56		standard	H Standard	7
L210S 12May	9.19		standard	H Standard	10
L210S 12May	0.1		standard	H Standard	0
L210S 12May	0.73	0.34	Control	1931	10
L210S 12May	2.21	1.02	Control	1931	20
L210S 12May	0.84	0.39	RLS	1637	10
L210S 12May	2.38	1.1	RLS	1637	20
L210S 12May	0.27	0.13	RLS	1162	10
L210S 12May	1.17	0.54	RLS	1162	20
L210S 12May	3.45	1.61	RLS	4954	10
L210S 12May	7.44	3.43	RLS	4954	20
L210S 12May	0.88	0.41	RLS	8	10
L210S 12May	2.57	1.19	RLS	8	20
L210S 12May	0.67	0.31	Control	3631	10
L210S 12May	2.12	0.98	Control	3631	20
L210S 12May	2.48	1.16	P1	Pooled	10
L210S 12May	6.57	3.03	P2	Pooled	20

Appendix
Section 2

Principles of End-point reverse transcription PCR

Microarray technology is a powerful technique that allows for the analysis of thousands of genes in a short time. The technique, however, is prone to several types of variability beyond biological differences in the population from which the sample is derived, including variability between sample preparations, labeling of samples, and hybridizations. For these reasons, microarray results routinely undergo verification, generally by reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR is more sensitive and easier to perform than other RNA analysis techniques, including Northern blots and ribonuclease protection assays. RT-PCR differs from the traditional PCR method of DNA fragment amplification in that the PCR is preceded by a reverse transcription (RT) reaction to produce cDNA from RNA and thus allow for analysis of gene expression. Traditional RT-PCR quantitation (referred to as end-point RT-PCR) relies on the use agarose gels for detection of amplification at the final phase (end-point) of the reaction, and size discrimination may therefore not be precise. Additionally, the end point is variable from sample to sample.

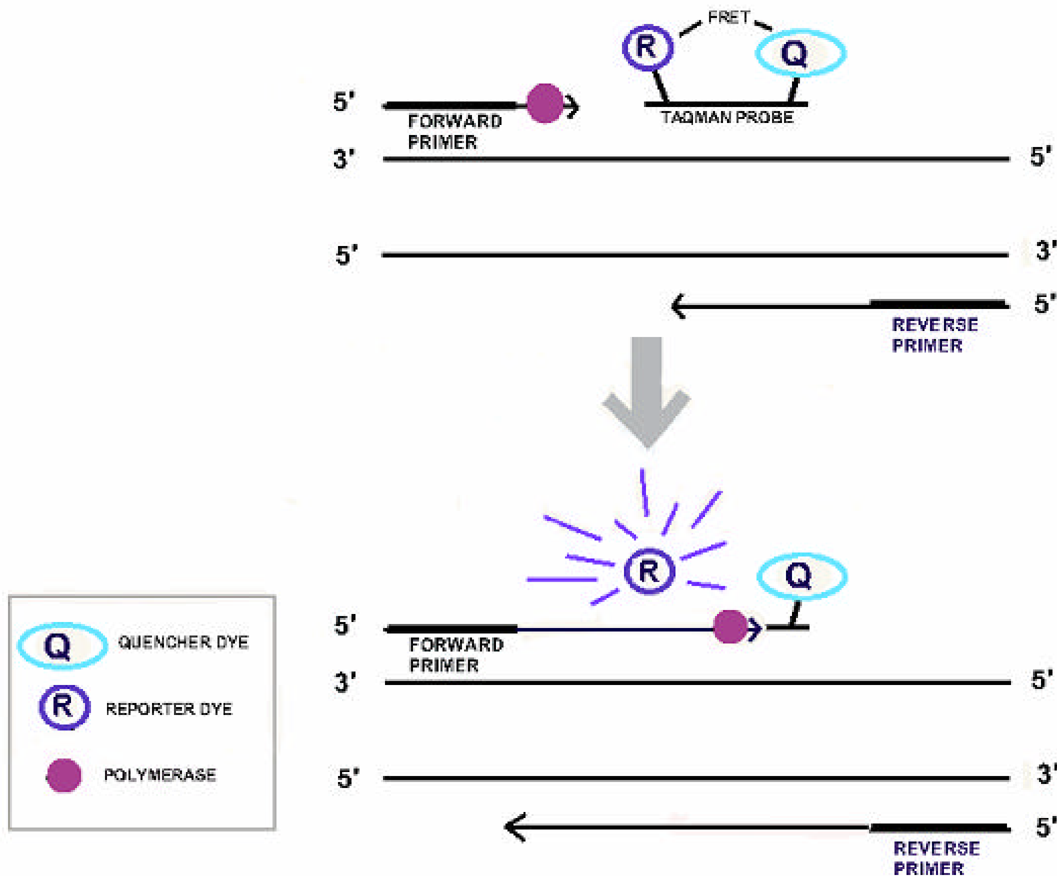
Principles of real-time PCR and system selection

Real-time RT-PCR quantitates reaction products in every cycle, resulting in a broad dynamic range, without the need for user intervention or replicates. There are currently four different systems widely available for real-time RT-PCR, differing by chemistry, quantitation methods, and instrumentation: Molecular Beacons, Scorpions, SYBR Green (Molecular Probes), and TaqMan (Applied Biosystems). All of these systems utilize a fluorescent signal for the

detection of the PCR product. Focus is on the latter two systems as they are readily available in the core facility at the Penn State College of Medicine (Note, however, that Scorpions and Molecular Beacons operate on the same principle as TaqMan; SYBR green is the only one of the four operating on a different principle).

The SYBR Green method relies on the fact that the SYBR Green signal shows little fluorescence in solution, emitting signal only after hybridizing to double-stranded DNA. While SYBR Green is inexpensive and sensitive, SYBR Green can bind to any double-stranded DNA in the reaction, including non-specific reaction products, resulting in an overestimation of actual target concentration. SYBR Green also requires a large degree of optimization, as the dye cannot differentiate specific and non-specific product.

TaqMan probes utilize Förster Resonance Energy Transfer (FRET) to generate a fluorescence signal. FRET couples a fluorescent reporter dye (6 -carboxyfluorescein (FAM) in the assays in this thesis) on the 5' end of an oligonucleotide and a quencher dye (6-carboxy-tetramethyl-rhodamine (TAMRA) in these assays) on the 3' end of the oligonucleotide. In the unhybridized state, the proximity of the fluorescent dye and the quenching dye prevents detection of probe fluorescence. The TaqMan probe is located between the two PCR primers and has a melting temperature 10°C higher than that of the primers (the temperature is increased in PCR to separate the double stranded DNA in each round of the amplification process). During replication, the 5'-3' exonuclease activity of the Taq DNA polymerase cleaves the TaqMan probe, separating the fluorescent and quenching dyes. The fluorescence increases in each cycle, proportional to the rate of probe cleavage.



PCR Quantitation

Quantitation for both end-point and real-time PCR can be either relative or absolute. Relative quantitation compares transcript amount across samples and yields an estimate of the relative expression of target RNA in the samples. This method is useful when analyzing changes in gene expression in a sample relative to an untreated control sample. Results are expressed as ratios of the gene signal to the internal control signal, and are often denoted as "fold change." A validation experiment must be run to show that the efficiencies of the target and endogenous control amplifications are approximately equal, but the need for a standard curve is eliminated (as is concern regarding dilution errors made in creating a standard curve) and therefore throughput is increased.

Absolute quantitation measures the specific amount of an mRNA sequence in a sample by interpolating its quantity from a standard curve. This is achieved by adding dilutions of a synthetic RNA to sample RNA replicates. The dilutions are co-amplified with the endogenous target, and the PCR product from the endogenous transcript is then compared to the concentration curve of the synthetic "competitor" RNA. Absolute quantitation is especially useful in settings where exact copy number is desired, as in the example of viral load. The absolute quantities of the standards must be known by independent means, generally by measuring concentration by A260 and converting copy number by using the molecular weight of the nucleic acid. The absolute quantitation technique also requires additional experiments to determine the range for the standard curve.

Statistical Analysis of Taqman Real Time PCR

Statistical interpretation of Real Time PCR data utilizes the comparative CT method. The intersection of the amplification plot and the threshold is defined as the cycle threshold (CT) value; that is, CT value represents the PCR cycle at which the software first detects an increase in the fluorescence of the reporter above baseline. The CT value is directly related to the amount of PCR product and therefore related to the initial amount of target present in the PCR reaction. The Assays by Design software (Applied Biosystems, Foster City, CA) is used to design oligonucleotide triplets (two primers plus a TaqMan probe) not otherwise available in the Applied Biosystems catalog. Amplification of all triplets can be performed with the same protocol and universal master mix. The delta CT represents the difference between the threshold cycle of the gene of interest (experimental or control sample) compared (normalized) to the corresponding endogenous housekeeping gene. The delta delta CT is the average difference

between the average delta CT of the experimental sample and the average delta CT for the corresponding control sample. The equation is:

[delta][delta]Ctsample = Avg[delta]CTsample - Avg[delta]CTreference, where [delta]CT sample is the CT value for a sample normalized to the endogenous housekeeping gene and [delta]CT reference is the CT value of the housekeeping gene-normalized calibrator.

The [delta][delta]Ctsample is then used to calculate expression fold value, as follows:

Expression fold value = 2 ^ - [delta][delta]Ctsample

The result is reported as an RQ value (relative quantity), which is actually the fold change of the sample. Results are displayed on an amplification plot displaying the generation of the reporter dye.

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