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Effects of iron status and development on ferroportin and hepcidin gene expression in rat brain

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EFFECTS OF IRON STATUS AND DEVELOPMENT ON FERROPORTIN AND HEPCIDIN GENE EXPRESSION IN RAT BRAIN

ABSTRACT

While iron is essential in living organisms, deficiencies or excesses can lead to pathological conditions such as iron deficiency anemia or hemochromatosis. Consequently, iron metabolism is tightly regulated by several factors. Ferroportin, the sole characterized mammalian iron exporter, and hepcidin, a liver produced peptide capable of degrading ferroportin, has recently been identified in the brain.

In the present study, the effect of development and iron status on ferroportin and hepcidin gene expression in the rat brain was investigated.

In the experiment investigating the effects of development, Wistar rats were killed after 2 weeks, 8 weeks and 8 months. The brain was microdissected into cerebellum, ventral tegmental area (VTA) and habenula.

In the experiment studying the effects of iron status, adult Wistar rats were subjected to iron deficiency ensuring a reduced iron access for the the fetus during the gestational period. The offspring of iron deficient dams were designated into two groups, a treatment group where pups received iron injections and a group receiving saline injections. At the age of 8 weeks, female rats were killed and key organs were harvested. The brain was dissected into samples of cerebral cortex, cerebellum, striatum and brain stem.

The results revealed that aging significantly increased brain iron concentrations with the highest amount in the cerebellum. Ferroportin gene expression in all brain areas declined significantly with aging despite an increase in iron. The presence of hepcidin mRNA in the rat brain was confirmed, however to a minimal extent. Furthermore, age had no significant effect on hepcidin gene expression.

Iron status was shown to have an effect on cerebral cortex iron content, although not significant. Ferroportin gene expression was significantly up regulated in the duodenum of iron deficient rats compared to rats receiving iron supplements. In the liver, ferroportin gene expression was vice versa to that of the duodenum. No significant alteration in ferroportin gene expression was observed in different brain areas of iron deficient, iron reverted and control rats. The level of hepcidin mRNA expression in the liver and duodenum, of rats receiving iron supplements compared to iron deficient rats, was significantly higher. Moreover, hepcidin expression was extremely low in all brain areas investigated despite differences in brain iron level.

In total, iron status and development have some effects on ferroportin and hepcidin gene expression and it seems that other factors, than brain iron content, might influence the expression of key iron transport molecules in the brain.

PREFACE

This thesis was written by Michael Winther Boserup during the 3rd and 4th semester of the Master of Science in Medicine with Industrial Specialisation program at the Department of Health Science and Technology, Aalborg University.

Notice that all abbreviations and the list of publications cited can be found at the end of the thesis. References are cited in square brackets, with author's last name and publication year.

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Indhold

1 INTRODUCTION

Iron is an essential metal involved in multiple physiological and biochemical processes throughout the body, including the brain. It plays a central role in the structure of hemoglobin and is a necessary constituent of iron-sulfur proteins, heme molecules and iron-dependent enzymes, which are utilized in the majority of oxidative reactions in the mitochondrial respiratory chain. Moreover, iron is involved in the regulation of cell mitosis (Bohnsack and Hirschi, 2004, Crichton, 2009).
In the brain, iron is vital for the synthesis of myelin-proteins and fatty acids in oligodendrocytes, and for the synthesis of neurotransmitters in neurons. Thus, emphasizing a continuous need for the brain to absorb iron from the circulation (Crichton, 2009, Rouault and Cooperman, 2006, Todorich et al., 2009).
A misbalance in iron homeostasis can be devastating, both when concentrations of iron is low and high. Iron deficiency is connected to several pathological conditions and accumulation of iron has been associated with hemochromatosis and production of free radicals that potentially leads to neuronal damage (Altamura and Muckenthaler, 2009, Chua et al., 2007).
According to the world health organization, anemia affects approximately 25% of the world population with the largest prevalence in preschool children and pregnant women (Benoist, 2008). Although the prevalence of anemia is significantly higher in undeveloped countries, it is an issue in the western world as well. In Denmark 9% of the whole population and 12.4% of pregnant women are anemic (Benoist, 2008). Iron deficiency is considered one of the largest contributing factors to anemia and can have severe effects on both cognitive and physical development in children and on physical performance in adults (Rodgers and Vaughan, 2002). Previously it was thought that the deleterious effects of iron deficiency were mediated through anemia. However, it is now known that even a small drop of iron levels in the central nervous system (CNS), which does not necessarily lead to anemia, alters the cerebral energy metabolism (Dallman, 1986).
Iron overload is characterized by excessive accumulation of iron in various tissues, resulting in tissue damage and ultimately organ failure. Iron overload can be caused by disorders of iron regulation proteins, as a consequence of dietary iron overload or because of blood transfusion (Crichton, 2009).
The most common form of iron overload is hereditary hemochromatosis, with a prevalence of 2 in 1.000 Caucasians (Pietrangelo, 2010). Furthermore, iron accumulation in the brain is theorized to play a role in both normal aging and in several pathological conditions affecting the CNS (Stankiewicz and Brass, 2009).
Iron metabolism is obviously tightly regulated in the human organism. This is consolidated by the delicate iron absorption and excretion which is responsible for adjusting iron homeostasis on the cellular and organ level.

1.1 IRON ABSORPTION AND METABOLISM

The amount of iron in the human body, under non-pathological conditions is ranging around 3-4 g (Baynes and Dominiczak, 1999). The exfoliation of epithelial cells in the gastrointestinal tract, urinary tract and on the integument results in a daily loss of about 0.5 mg – 2.0 mg (Sharp and Srai, 2007). Thus, a dietary iron up-take is required.

1.1.1 DIETARY IRON IMPORT

Iron is absorbed in the proximal part of the small intestine, especially in the duodenum (McKie et al., 2001). A simplified illustration of iron absorption and metabolism is showed in Figure 1 (Anderson et al., 2009).

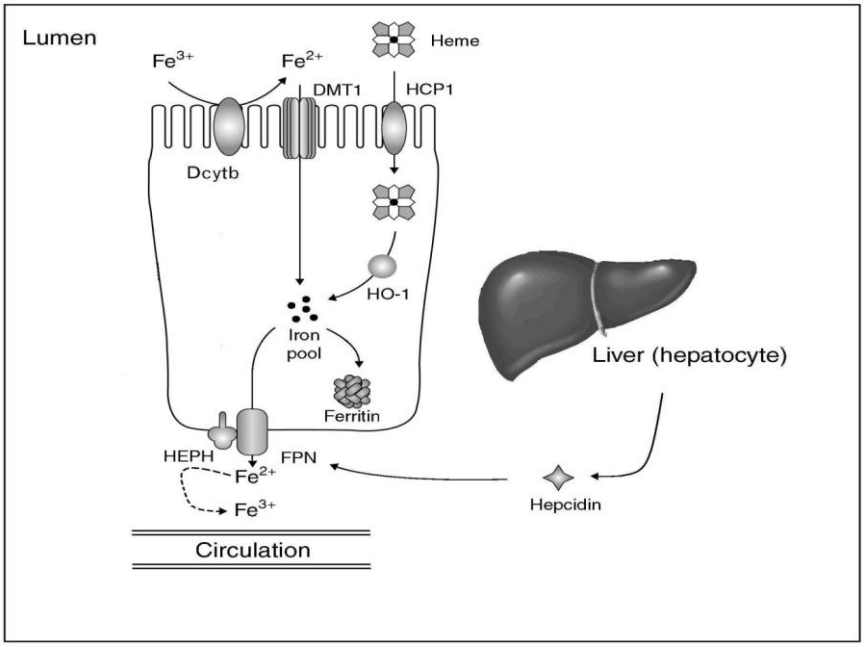

Figure 1: Iron is present in the lumen as Fe^{3+} and is reduced to Fe^{2+} by Dcytb located on the brush border of duodenal enterocytes. Fe^{2+} is then transported into the cytoplasm by DMT1. Heme bound iron is transported to the cytoplasm via the carrier protein HCP1, whereupon the iron is stored in vesicles and oxidized by HO-1, resulting in iron separation. Free Fe^{2+} accumulates in a common pool and is stored by the intracellular protein ferritin. Fe^{2+} is exported out of the cell via Fpn. Before binding to transferrin, Fe^{2+} is oxidized to Fe^{3+} probably by HEPH. Hepcidin is a hormone responsible for the internalization and degradation of Fpn. Dcytb: duodenal cytochrome b. DMT1: divalent metal transporter 1. HCP1: heme carrier proteins 1. HO-1: heme oxygenase 1. HEPH: hephaestin. Fpn: ferroportin. Illustration modified from Anderson et al, 2009.

Dietary iron is available in two basic forms, either bound to heme or in a non-heme form. Non-heme bound iron is obtained from vegetables, cereals and fruits with a bioavailability of 1%-10% and heme bound iron from meat products with a bioavailability of 20%-30% (Sharp and Srai, 2007).
Non-heme iron entering the duodenum is profoundly available on the ferric form (Fe^{3+}), which is considered non-bioavailable. In order for the iron to be absorbed, it must be converted from Fe^{3+} to ferrous iron (Fe^{2+}). Several dietary components have such reducing capabilities, including ascorbic acid

(Dorey et al., 1993, Han et al., 1995). Moreover, the brush border surface of duodenal enterocytes contains the enzyme duodenal cytochrome b (Dcytb), which possess ferric reducing characteristics (McKie et al., 2001). Subsequent to the reduction by either Dcytb or dietary components, Fe^{2+} is transported across the membrane of the enterocyte via the divalent metal transporter 1 (DMT1) (Gunshin et al., 1997). It is suggested that the low pH level in the proximal duodenum and the acidic microenvironment at the enterocyte brush border, facilitate iron uptake across the membrane by stabilizing ferrous iron and by providing protons essential in transmembrane movement (Sharp and Srai, 2007, Tandy et al., 2000).
The mechanism behind absorption of heme iron is not fully understood. An early theory proposes that heme iron is absorbed via endocytosis as an intact molecule (Hallberg, 1981). Supporting this hypothesis, the duodenal enterocytes have been shown to express high amounts of heme carrier proteins (HCP1) which are important for influx of heme molecules (Shayeghi et al., 2005).
Following absorption, heme is bond to vesicles that probably interacts with heme oxygenase 1 (HO-1) causing iron to separate from the heme molecule and accumulate in a common intracellular pool (Parmley et al., 1981). If iron demand is low, a large amount of the accumulated iron is stored in intracellular protein complexes known as ferritin (Arosio and Levi, 2002). Ferritin is able to store up to a few thousand iron atoms by binding Fe^{2+} to a ferroxidase site (Arosio and Levi, 2002). Fe^{2+} is then oxidized to Fe^{3+} and aggregated in an iron core (Arosio and Levi, 2002).
Due to the rapid turnover of enterocytes, ferritin is constantly lost into the intestinal lumen as aging cells are sloughed. This is believed to be important for maintaining iron homeostasis (Hunt and Roughead, 1999).

1.1.2 Iron export to the circulation

Cellular iron export involves a two-step process, starting with ferroportin that transports iron to the basolateral surface of enterocytes, if iron demand is high (Donovan et al., 2000).
Ferroportin is internalized and degraded by hepcidin, a hormone produced by the liver (Park et al., 2001). Before iron can be released from ferroportin and bind to transferrin it has to be oxidized by a ferroxidase to Fe^{3+} (Vulpe et al., 1999).
Studies have demonstrated that in the intestine this oxidation is facilitated by the ceruloplasmin homologue hephaestin (HEPH) (Chen et al., 2004). The oxidized Fe^{3+} binds to an iron-carrying plasma protein, apotransferrin, resulting in a conformational change to transferrin (Tf), before being transported via the circulation to other tissues. Tf interacts with the transferrin receptor (TfR) on the cell membrane internalizing iron via endocytosis of the Tf–TfR complex (Cheng et al., 2004).

1.2 Brain iron metabolism

Transferrin bound iron (Tf-Fe) is hydrophilic and therefore unable to pass the blood brain barrier (BBB) passively (Moos et al., 2007). Consequently, the brain expresses the transmembrane molecule transferrin receptor 1 (TfR1) on the luminal side of its brain capillary endothelial cells (BCECs), which can move iron across the BBB (Angelova-Gateva, 1980).
Iron uptake is predominantly achieved via TfR1 interaction. However, a small fraction of total iron uptake occurs through the choroids plexuses. The translocation of Tf-Fe is a multistep process starting with the attachment of the Tf-Fe complex to the extracellular segment of the TfR1 followed by endocytosis and formation of an endosome containing the Tf-Fe complex. The endosomal environment is

slightly acidic, causing Fe^{3+} to be disassociated from the transferrin molecule and reduced to Fe^{2+}. Ferrous iron is then liberated from the endosome, probably by DMT1, which transports Fe^{2+} to the cytosol in exchange of two protons (Gunshin et al., 1997). However, the release of iron from the endosome via DMT1 is believed only to be important for the BCECs own iron requirements and is not associated with abluminal export of iron from the BCECs. (Benarroch, 2009, Moos et al., 2007)
The mechanism by which unbound Fe^{2+} is transported from BCECs to the extracellular fluid is a matter of controversy (Benarroch, 2009, Moos et al., 2007). One possibility is that the unbound Fe^{2+} is transported from the endosome to the cytosol by the action of DMT1. Fe^{2+} in the cytosol is then exported into the extracellular fluid by action of ferroportin (Benarroch, 2009). However, there is some disagreement as to whether DMT1 and ferroportin are expressed on BCECs and how they contribute to iron transport across the BCECs (Benarroch, 2009, Moos et al., 2007). Alternatively, it has been proposed that the Tf-TfR1 complex is transported within endosomes directly from the luminal side to the basolateral surface, where endosomes fuse with the cell membrane and release unbound ferrous iron into the extracellular fluid of the brain. Ceruloplasmin, expressed in astrocyte end-foot processes, oxidizes newly released Fe^{2+} to Fe^{3+}, which binds to apotransferrin. Ferrous iron may also bind to ATP or citrate released from astrocytes and transported in form of non-transferrin-bound iron (Benarroch, 2009, Moos et al., 2007).
The clearance of iron from the brain is facilitated via absorption of cerebrospinal fluid at the arachnoid villi (Moos and Morgan, 1998). At the cellular level, the handling of iron in the brain is complex due to the fact that some cell types seems to have an independent regulation of iron metabolism trough uptake, storage and excretion (Moos et al., 2007).

1.3 Iron export proteins

1.3.1 Ferroportin

The ferroportin molecule (also known as Ireg-1 or Metal Transporter Protein 1; gene symbol Slc40a1) is capable of transporting ferrous iron across cellular membranes. It was initially detected in duodenal enterocytes, placental trophoblasts and macrophages of the liver and spleen (Donovan et al., 2000, Haile, 2000, McKie et al., 2000).
There is little genetic difference between human, rat and mouse ferroportin with a sequence similarity of 90-95 %. The ferroportin gene spans 20 kb and contains 8 exons and encodes a protein of approximate 62 kDa (Abboud and Haile, 2000, Rice et al., 2009).
Ferroportin is expressed in numerous cells and especially enterocytes, hepatocytes and macrophages show high levels of expression (Zhang et al., 2009).
In the brain, the expression of ferroportin has been detected in discrete brain regions of the rat, mouse and man, revealing a neuronal distribution in the cerebral cortex, thalamus, brain stem, hippocampus and cerebellum (Boserup et al., 2011, Burdo et al., 2001, Koeppen et al., 2007, Wu et al., 2004). While the expression of ferroportin in neurons is undisputable, the distribution in other non-neuronal cells is still debated. Especially endothelial cells and choroid plexus epithelial cells is a matter of contradiction (Boserup et al., 2011, Rouault et al., 2009).
Ferroportin is regulated at the transcriptional, translational and post translational level. The ferroportin mRNA contains an iron-responsive element (IRE) in its 5' untranslated region (UTR) and has an expression pattern similar to that of ferritin (Abboud and Haile, 2000). The effect of IREs is mediated

by iron-responsive proteins (IRPs) that are regulated by cellular iron status. When intracellular iron levels are low, the IRP-IRE interaction enables cells to replenish cytosolic iron by increasing iron uptake through DMT1 and TfR1, while simultaneously decreasing iron sequestration in ferritin, and suppressing iron export through ferroportin (Zhang et al., 2009, Levenson and Tassabehji, 2004). However, enterocytes and erythroid precursor cells have been shown to utilize an alternative ferroportin transcript, ferroportin1B, which lacks the IRE, making it possible to explain the high expression of ferroportin in the duodenum under iron deficient conditions (Zhang et al., 2009).
Post-translational regulation is mediated by hepcidin, a small peptide hormone produced in the liver. Hepcidin binds to ferroportin, inducing its internalization and degradation by interaction with Janus kinase2 (De Domenico et al., 2009).

1.3.2 HEPCIDIN

The hepcidin peptide is derived from an 84 amino acid precursor peptide called pro-hepcidin. In humans, it is produced as a 20 or 25 amino acid peptide. Homologous genes have also been identified in pig, rat, mouse flounder and long-jaw mudsucker. The human hepcidin gene (HAMP) and the murine HEPC-1-gene consists of three exons and two introns (Park et al., 2001).
Hepcidin is expressed predominantly in the liver, where it is synthesized and released into the blood, but is also detectable in heart, skeletal muscles, lung and pancreas (Pigeon et al., 2001).
Hepcidin has been identified in the murine brain, with distinct expression in the cerebral cortex, hippocampus, striatum, diencephalon, midbrain, cerebellum and pons (Wang et al., 2010, Zechel et al., 2006). On the cellular level, the hepcidin protein has been detected in both neurons and glia cells (Zechel et al., 2006).
Hepcidin expression is iron sensitive, resulting in a decrease of hepcidin in conditions with low iron and an increase in iron overload conditions. This has been shown on hepcidin mRNA in iron-overload and iron deficient mice (Pigeon et al., 2001). Hepcidin is also regulated by a number of proteins expressed in hepatocytes, including Tf, Bone morphogenic protein 6 (BMP6), hemojuvelin (HJV), TfR2 and the hereditary hemochromatosis protein called HFE. Moreover, hypoxia, erythroid factors and inflammation have a robust regulatory effect on hepcidin expression regardless of iron status (Ganz, 2011).

1.4 CONSEQUENCES OF IRON DEFICIENCY

Iron is a central structural part of hemoglobin, which consists of four globin subunits and four heme subunits with an integrated Fe^{2+} molecule, each capable of binding one carbon oxide or one oxygen molecule (Baynes and Dominiczak, 1999). Iron deficiency is most commonly caused by an inadequate nutritional intake or blood loss due to menstruation.
Clinically, iron deficiency can be detected by measuring hemoglobin, hematocrit, transferrin iron saturation or mean corpuscular hemoglobin concentration. The general symptoms of iron deficiency are fatigue, dizziness, hypothermia and paleness (Schroeder, 2004).
Iron deficiency during human development leads to long-term impairments in intelligence and cognitive ability in humans (Georgieff, 2008), as well as a decrease in fine motor performance (Lozoff et al., 1991). This indicates that learning and cognitive centers in the brain, such as the hippocampus, may be compromised in the case of iron deficiency during neural development.
The consequences of anemia and iron deficiency in infants and children are often long-term. Several

studies have assessed overall developmental outcomes, showing long term effects of iron deficiency on a range of milestone perimeters. Furthermore, Iron deficiency has also shown to have a negative effect on IQ (Lozoff, 2007)

1.5 IRON DEFICIENCY AND THE BRAIN

The iron demand of the brain is highest during the growth spurt beginning in the last trimester, until the age of 2 years in humans. In rats, the growth spurt starts at the day of birth until weaning, corresponding to the last trimester in humans (McCann and Ames, 2007). Within this period of development, where neuronal processes remain capable of plasticity, a window of opportunity exists (Beard et al., 2007, Lozoff et al., 2006).
Iron is crucial during CNS development, because of its central role in neurotransmitter and fatty acid synthesis, synaptogenesis, dendritic arborization and myelination (LeBlanc et al., 2009).

1.5.1 MONOAMINES

The most studied and well documented monoamine affected by iron deficiency is dopamine (DA), but altered metabolism of other monoamines such as serotonin (5-HT) and noradrenaline (NA) has also been observed. Iron deficiency is thought to alter factors that affect monoamine homeostasis, such as iron-dependent enzymes used in neurotransmitter synthesis, the concentration of specific neurotransmitters and the density of both monoamine receptors and transporters (Felt et al., 2006).
DA is the predominant neurotransmitter of the mammalian extrapyramidal system and of several mesocortical and mesolimbic neuronal pathways (Goodman et al., 2008). Receptors for DA (D_1 and D_2) are widely distributed in the brain, where especially the striatum contains many receptors for DA. The striatum is a part of the basal ganglia, which is richly innervated by dopaminergic fibers and related to cognitive and emotional processes, memory, motivational behavior and motor function. The amount of dopaminergic fibers in the striatum is closely correlated to the amount of iron (Lozoff and Georgieff, 2006). Tyrosine hydroxylase is a key enzyme in the synthesis of DA and NA. This enzyme is iron-dependent and therefore not capable of full enzymatic function under iron deficient circumstances (Youdim, 2008, Beard et al., 2003).
Iron deficiency influences the mechanism responsible for the recycling of DA. The dopamine transporter (DAT) imports DA from the extracellular fluid into the presynaptic neurons, where it can be stored in vesicles for later utilization. In iron deficient conditions, the density of DAT is reduced, which leads to a decreased re-uptake of extracellular DA (Erikson et al., 2000). In addition, iron deficiency alters D_1 and D_2 densities, resulting in changed monoamine metabolism (Beard and Connor, 2003).

1.5.2 SYNAPSES

Synapses and dendrites are important for the formation of neuronal circuits. Synapse formation occurs either by arrangement of synaptic boutons along the axon shaft or by formation of boutons at the end of axon branches (Jin, 2005). It is suggested that synthesis of cellular signalling molecules, such as protein kinase C (Schrenk et al., 2002) and glutamate (Wilson and Keith, 1998), which has the potential of stimulating dendritic growth, is compromised by iron deficiency (Rao et al., 2003). Several neurotrophins important for synapses and dendritic spine maturation have been discovered, including brain derived neurotrophic factor (BDNF). BDNF is multifunctional and regulates neurogenesis, neuronal

survival, dendritic growth and branching, and plasticity across the life span (Vicario-Abejon et al., 1998).
It has been demonstrated that fetal-neonatal iron deficiency lowers the level of BDNF mRNA expression in the rat hippocampus, suggesting that iron deficiency has a detrimental effect on synaptogenesis and alters synaptic and dendritic morphology (Tran et al., 2008). Moreover, iron deficiency induces an up-regulation of other neurotrophic factors such as nerve growth factor (NGF), epithelial growth factor (EGF) and glia-derived neurotrophic factor (GDNF). This indicates a utilization of other signalling pathways as compensation for a compromised BDNF signalling pathway (Tran et al., 2008).
Results from electrophysiological studies, investigating synaptic transmission, supports that iron deficiency alters synaptic and dendritic structure and function. Perinatal iron deficiency leads to a significant reduction in synaptic transmission in hippocampal areas, compared to iron sufficient controls (McEchron and Paronish, 2005).

1.5.3 Oligodendrocytes and myelination

Studies have demonstrated that decreased iron availability is strongly associated with hypomyelination caused by changed metabolic activity in oligodendrocytes (Todorich et al., 2009).
Oligodendrocytes are the cells in the CNS that stain most strongly for iron, even in areas with high iron concentration, e.g. cerebellar nuclei, substantia nigra and the striatum (Todorich et al., 2009). Oligodendrocytes produce myelin for insulation of neuronal axonal processes, which enables saltatory conduction, and the importance of iron in numerous processes regarding the production of myelin cannot be understated (Morath and Mayer-Proschel, 2001).
Enzymes used in cholesterol and fatty acid synthesis of myelin are highly iron dependent, and thus affected by iron deficient states. Furthermore, the interaction of iron in ATP production as a cofactor in cytochrome A, B and C is cardinal, due to the fact that oligodendrocytes are the most metabolically active cells in the brain. During iron deprivation the metabolic demand in oligodendrocytes is insufficient, resulting in limited myelin production (Todorich et al., 2009).
Cholesterol and lipid synthesis occurs at a higher rate in oligodendrocytes than any other cell type in the brain. Iron-dependent enzymes involved in lipid synthesis (fatty acid desaturase) and degradation (lipid dehydrogenases) are under normal circumstances present in substantial amounts in oligodendrocytes. Under iron deficient conditions, the amount of enzymes decline leading to a reduced oligodendrocyte functionality (Connor and Menzies, 1996). Beard and colleagues have investigated the effect of iron deficiency on the myelin production of oligodendrocytes, by measuring 2´, 3´-cyclic nucleotide 3´-phosphohydrolase (CNPase) and myelin basic protein (MBP). Their results showed that at iron deficient conditions, CNPase, cytochrome C and MBP were significantly reduced compared to controls (Beard et al., 2003).
The neurophysiological outcomes of hypomyelination caused by iron deficiency have been studied, by measuring auditory brainstem response and visual evoked potentials in six months old infants with iron deficiency anemia. Results showed a significant difference between the iron deficiency anemia infants and controls in these neurophysiological parameters, and it was concluded that hypomyelination was caused by the lack of iron. Though the children were treated, with oral iron tablets a significant difference still existed, providing evidence that the damage caused by the lack of iron can be long lasting or irreversible (Algarin et al., 2003).

1.6 CONSEQUENCES OF IRON ACCUMULATION

Iron overload is characterized by excessive accumulation of iron in various tissues, resulting in tissue damage and ultimately organ failure. Iron overload can be caused by disorders of iron regulation proteins, dietary iron overload or blood transfusion (Crichton, 2009). Some of the most prominent disorders assicociated with iron accumulation are listed in Table 1 (Pietrangelo, 2007).

Table 1: Classification of disorders associated with iron accumulation

Iron accumulation disorders	
Hereditary	**Acquired**
Hemochromatosis	Dietary
Ferroportin disease	Parenteral and transfusional
Aceruloplasminemia	Anemia of inflammation
Atransferrinemia	Acquired iron-loading anemias
Friedreich's ataxia	Long-term hemodialysis
Hereditary iron-loading anemias	Chronic liver disease

Hereditary hemochromatosis is closely linked to hepcidin and the most common iron overload disorder.

1.6.1 HEMOCHROMATOSIS

Hemochromatosis, also known as classical, idiopathic and hereditary hemochromatosis, is a disorder where genetic mutations lead to iron overload. Clinical symptoms and genetic mutations, divide the disease into four different classifications.

Type 1 hemochromatosis is caused by a mutation on chromosome 6 in the HFE gene and is the far most common iron-overload disorder, representing more than 90 % of the genetic disorders (Brissot et al., 2008). The HFE gene is involved in the regulation of hepcidin and mutations in this gene leads to increased iron absorption in the intestine and subsequent iron overload due to inadequate hepcidin synthesis. Clinically, the patients can present adrenal insufficiency, heart failure, cirrhosis or diabetes, with fatigue, malaise and a characteristic slate-gray color of the skin as the most common symptoms (Pietrangelo, 2010).

Type 2 hemochromatosis, also called juvenile hemochromatosis, is a rare but particularly severe form of hemochromatosis occurring typically in the first to third decades of life. It exists in either type 2A or 2B. Type 2A is a consequence of a mutation on chromosome 1 in the HFE2 gene which encodes the protein hemojuvelin. It is theorized that the function of the HFE2 gene is to modulate hepcidin expression (Papanikolaou et al., 2004).

Type 2B is caused by a mutation of the hepcidin antimicrobial (HAMP) gene on chromosome 19. Cardiac and endocrine dysfunctions are typical in type 2 hemochromatosis, because of iron accumulation in parenchymal cells, in particular liver and pancreas, caused by increased amounts of hepcidin

(Roetto et al., 2003).
Type 3 hemochromatosis is related to mutation in the TfR2 gene. As a consequence of this mutation TfR2 is inactivated, mimicking the clinical features of type 1 hemochromatosis (Roetto et al., 2001, Brissot et al., 2008).
Type 4 hemochromatosis, also known as ferroportin disease, is caused by a mutation of the SLC40A1 gene, coding for ferroportin, which is located on chromosome 2. It is subdivided in type A and B and is the only form of genetic iron overload disease with dominant pattern of inheritance. Type A results in normal or low plasma transferrin saturation and macrophage iron deposition. Type B is comparable with type 1 and 3 hemochromatosis, with increased plasma transferrin saturation and parenchymal iron deposition resulting in cardiomyopathy, arthropathy, and liver fibrosis or cirrhosis (Brissot et al., 2008).

1.6.2 Diseases of neurotoxicity and aging

Within the living organism, iron can be found on its reduced Fe^{2+} form and its oxidized Fe^{3+} form. These chemical properties are cardinal to a plethora of biological functions, but can induce toxicity if iron is unshielded. Excess free iron is involved in the production of damaging free radicals. Free radicals are a product of the Fenton reaction (Figure 2), which catalyzes the conversion of reactive oxygen species (ROS) to the highly reactive hydroxyl radical (OH●) damaging proteins, lipids and DNA (Altamura and Muckenthaler, 2009).
In nonpathological conditions, hydrogen peroxide is removed by catalase and glutathione peroxidases storing iron as ferritin preventing the formation of free radicals. Under pathological conditions, these mechanisms are compromised, making the cell more prone to oxidative stress (Berg and Youdim, 2006).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH^-$$

Figure 2: Fenton reaction. Ferrous iron (Fe^{2+}) is oxidized by hydrogen peroxide (H_2O_2) to ferric iron (Fe^{3+}), a hydroxyl radical (OH●) and a hydroxyl anion (OH^-).

Increases in metal ions may play a prominent role in the neurodegenerative process. Several studies have demonstrated that iron concentrations increase in the brain with normal aging (Brass et al., 2006, Focht et al., 1997).
Brain iron content accumulates during the first three decades of life, plateaus for the next three decades, and then increases gradually after the sixth decade of life (Stankiewicz and Brass, 2009).
Iron distribution in the brain is somewhat heterogeneous, especially in the adult brain where high concentrations of iron is seen in the nucleus rubor, nuclei cerabelli, substantia nigra, nucleus accumbens and portions of the hippocampus. Iron is contained in iron pools consisting of enzymes and structural proteins, but also in ferritin and transferrin. On the cellular level, iron is mainly located in oligodendrocytes and microglia and is amongst others utilized in the process of ATP production, myelin synthesis and neurotransmitter metabolism (Aoki et al., 1989, Beard and Connor, 2003). Iron levels remain constant in oligodendrocytes with age, whereas increases in iron concentration are observed in microglia and astrocytes of the elderly. In older brains, morphologically abnormal microglia are more likely to stain ferritin positive, suggesting that iron exposure over time can lead to degeneration (Lopes et al., 2008).

Neuropathological studies, animal models and in-vitro experiments have revealed that many neurodegenerative diseases are associated with increased brain iron deposition. Table 2 gives an overview of some of the most important (Stankiewicz and Brass, 2009).

Table 2: Neurological disorders associated with brain iron increase

Neurological disorders
Aceruloplasminemia
Alzheimer's disease
Friedreich's ataxia
Huntington's disease
Multiple sclerosis
Neuroferritinopathy
Parkinson's disease

Alzheimer's disease and Parkinson's disease typically affect the elderly and several connections with an imbalance in iron metabolism have been established, making it interesting to investigate.

Alzheimer's disease

It is estimated that 24 million people worldwide have dementia, with Alzheimer's disease as the main contributing factor (Ferri et al., 2005). The clinical manifestation of Alzheimer disease is dementia that typically begins with inability to acquire new memories, observed as difficulty in recalling recently observed events. As the disease slowly advances symptoms include irritability, confusion, mood swings, long-term memory loss and language disturbance, ultimately leading to death (Ballard et al., 2011, Bird, 1993). The typical clinical duration of the disease is eight to ten years (Bird, 1993).
Alzheimer's disease is hallmarked pathoanatomically, by senile plaques within the brain, proteinaceous deposits mainly composed of extracellular insoluble amyloid-β peptide, as well as neurofibrillary tangles created by the hyperphosphorylation of the microtubule associated protein tau that aggregates and causes microtubule collapsing (Altamura and Muckenthaler, 2009). The exact cause of Alzheimer's is not known, however genetics is estimated to account for 70% of the risk (Ballard et al., 2011).
Accumulation of iron in the brain, particularly in cells that are associated with senile plaques, is a consistent observation (Honda et al., 2004). Furthermore, considerable amounts of iron depositions have been demonstrated in the cerebral cortex, hippocampus and nucleus of Meynert (Zhu et al., 2007).
Amyloid-β binds iron, thereby increasing the toxicity of the peptide. This has been demonstrated in vivo, where injection of iron with amyloid-β in the adult rat brain, caused a significant higher neuronal damage than injections with amyloid-β alone (Honda et al., 2004).
Oxidative stress and Alzheimer's disease is closely linked, as it has been shown in postmortem brains of Alzheimer's disease patients, where elevated activities of antioxidant proteins such as glutathione reductase, glutathione peroxidase, superoxide dismutase, and catalase was found (Pappolla et al., 1992). Oxidative stress may be generated by the redox-active iron that is closely associated with the amyloid-β and the neurofibrillary tangles deposits (Altamura and Muckenthaler, 2009). These findings suggest that iron accumulation, might be involved with the pathogenesis of Alzheimer's disease.

Parkinson's disease

The prevalence of Parkinson's disease worldwide is estimated to 4 million people (Stoessl, 2011). Parkinson's disease is caused by a selective loss of the dopaminergic neurons of the substantia nigra. Loss of 50–70% of the approximately 450,000 dopamine producing cells results in the typical clinical symptoms of bradykinesia, dyskinesia, rigidity, and tremor. Furthermore, some patients present psychiatric manifestations, which include depression and visual hallucination. Dementia eventually occurs in at least 20% of cases (Thomas and Beal, 2007).

Although the etiology of Parkinson's disease is unknown, mutations have been identified in the parkin, PINK1, DJ-1 and α- synuclein genes, respectively (Pankratz and Foroud, 2007, Altamura and Muckenthaler, 2009). The molecular mechanisms thought to be responsible for development of Parkinson's disease include oxidative damage, mitochondrial dysfunction, abnormal protein accumulation and protein phosphorylation, all compromising dopamine neuronal function and survival.

A characteristic of Parkinson's disease is the presence of intracellular, eosinophilic proteinaceous aggregates called Lewy bodies, which are composed mostly of a-synuclein, but also contain ubiquitin, tyrosine hydroxylase and IRP 2 (Crichton et al., 2011). Lewy bodies are found within dopaminergic neurons, axons and synapses of the substantia nigra. Interesting, multiple studies have now shown that iron promotes the aggregation of α-synuclein, creating a possible link between iron accumulation and Parkinson's disease. Furthermore, increased iron has been reported in in the substantia nigra, and on the cellular level in astrocytes and neurons (Gaylin et al., 1999, Schipper et al., 1998). Iron is also found to accumulate within Lewy bodies in the brains of Parkinson's disease patients (Takanashi et al., 2001).

2 MATERIALS AND METHODS

2.1 EXPERIMENTAL DESIGN

While iron is essential in living organisms, deficiencies or excesses can lead to pathological conditions such as iron deficiency anemia or hemochromatosis. This project seeks to investigate the effects of development and iron status, primarily on brain iron efflux and regulation. Thus, ferroportin and hepcidin, two cardinal players in iron transport is investigated in relation to development and iron status in Wistar rats.

2.1.1 IRON STATUS EXPERIMENT

Adult female rats were subjected to iron deficiency, by exsanguination and restriction of iron in the diet before pregnancy, ensuring reduced iron access for the the fetus during the gestational period. The level of iron deficiency was observed trough weight measurements, hemoglobin analyses and visual differences (Figure 3). To examine if the consequences of iron deficiency were reversible, half of the pups from the iron deficient dams, were injected with iron supplements (isomaltoside 1000) whereas the other half was injected with saline. At the age of 8 weeks, female rats were killed and key organs were harvested. The brain was dissected into samples of cerebral cortex, cerebellum, striatum and brain stem. Liver and duodenum was extracted, because of their high content of ferroportin and hepcidin. The frozen preparations were used in biochemical analyses to measure total iron content and mRNA expression of ferroportin and hepcidin.

Figure 3: The physical appearance of an ID rat (left) and a control rat (right).

2.1.2 DEVELOPMENT EXPERIMENT

Normal Wistar rats were killed after 2 weeks, 8 weeks and 8 months. The brain was microdissected into cerebellum, ventral tegmental area (VTA) and habenula. The frozen brain preparations were used in biochemical analyses to measure total iron content and mRNA expression of ferroportin and hepcidin. Furthermore, western blot analyses were conducted to measure the semi-quantitative expression of the ferroportin protein, but due to antibody difficulties the results were inconclusive and not included in the thesis.

Table 3 gives an overview of the development and iron status experiments, in relation to group, tissue and analyses conducted.

Table 3: Overview of the development and iron status experiment.

Development		
Group	**Tissue**	**Analyses**
2 week	Cerebellum	Total iron content
8 week	VTA	RT-PCR
8 month	Habenula	RT-qPCR

Iron status		
Group	**Tissue**	**Analyses**
Control	Cerebral cortex	Total iron content
ID	Cerebellum	RT-PCR
ID + Fe	Striatum	RT-qPCR
	Brain stem	
	Duodenum	
	Liver	

2.2 ANIMALS AND DIET

During the experiment rats had access to water and food *ad libitum.* They were housed in 48cm x 37.5cm x 21cm cages (1500U Eurostandard Type IV S, Scanbur A/S, Karlslunde, DK) at the Animal Department of Aalborg Hospital, Aalborg, DK. The rats were housed under constant temperature and humidity conditions and kept on a 12 hour light/dark cycle. All procedures concerning animals in this study were approved by the Danish Experimental Animal Inspectorate under the Ministry of Justice.

2.2.1 DEVELOPING RATS

Rats (n=15) of the Wistar strain (Taconic, Ry, DK), were kept on a normal diet (1214 FORTI breeding diet, Altromin Spezialfutter, DE) and randomly assigned into three groups of different age. 2 weeks old rats (n=5), 8 weeks old rats (n=5) and 8 months old rats (n=5).

2.2.2 IRON STATUS RATS

Female rats (n=14) of the Wistar strain (age: 12 weeks) were purchased from a commercial supplier (Taconic, Ry, DK) and kept on a normal diet (1214 FORTI breeding diet, Altromin Spezialfutter, DE) the first week after arrival. The rats were placed in individual cages, composed of a plastic bottom and sealed with a metal lid. Cages were filled with a layer of sawdust, a small pile of hay to use as nesting material and a transparent red cylinder. A week after arriving, the rats were weighed (240g – 260g).

The rats were randomly assigned into an iron deficient (ID) group (n=9) and a control group (n=5). Iron deficiency was induced by collecting 2.0 ml (equals 1% of total body weight) of blood. This was done by anaesthetizing the rats with Hypnorm (0.315 mg/ml fentanyl citrate, VetaPharma, UK) – Dormicum (5 mg/ml, Hameln Pharmaceutical, Gloucester, UK) diluted in a saline solution (mixture proportion: 1:1:2), at a dose of 0.15 – 0.2 ml per 100g. Afterwards blood was either collected by left ventricle heart puncture (n=8) or by tail vene puncture (n=2). One rat died following the heart puncture and was replaced by a rat from the control group. To maintain iron deficiency the ID rats received a special controlled diet low in iron (<10mg/kg) (C 1038 iron deficient diet, Altromin Spezialfutter, DE), whereas control rats were kept on a normal diet. Two days post blood collection, female rats (n=13) were mated with male Wistar rats (n=13) fed a normal diet. Male rats were placed with female rats for 8 days to ensure pregnancy.

The offspring (n=174) was born approximately after three weeks of gestation. Iron deficient rats (n=9) gave birth to 122 pups (12-15 pups/litter), whereas the iron sufficient rats (n=4) gave birth to 52 pups (10-15 pups/litter). Dams with matching pups were divided into 3 groups:

1. Iron deficient pups receiving saline (ID) (n=68).
2. Iron deficient pups receiving isomaltoside 1000 (ID + Fe) (n= 54).
3. Iron sufficient pups receiving saline (control) (n=42)

Iron isomaltoside 1000 (PharmaCosmos, Holbaek, DK) was diluted in a saline solution and given as subcutaneously neck injections in the ID + Fe group. Injections of iron dextran began at postnatal day (p) 1-4 at a dose of 45 mg Fe/kg body weight. Rat pups in the ID and control group were injected with saline solution instead of iron isomaltoside 1000 from P3-P6. All injections and weight measurements were continued every 3-4 days.

2.3 TISSUE PREPARATION

The rats were anaesthetized with a high dose of Hypnorm–Dormicum diluted in a saline solution. When they reached unconsciousness, the brain was removed from the cranium and quickly stored on dry ice in 50 ml nunc tubes. The organs were subsequently stored at -80°C.
The brain tissue of the developing rats (n=15) was dissected on ice under a dissecting microscope to isolate the cerebellum, the ventral tegmental area (VTA) and the habenular region. The latter is situated in the region of the dorsal thalamus.
Iron status rats (n=15) brain tissue was dissected into cerebral cortex, cerebellum, striatum, brain stem. Furthermore, the right liver lobe and the proximal part of the duodenum was dissected and used for analysis.

2.4 BIOCHEMICAL ANALYSES

The following methods were applied in both the development and iron status experiment.

2.4.1 PCR ANALYSES

Total RNA was extracted from brain, liver and duodenum tissue with NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Ger) and cDNA synthesis conducted with 1 µg RNA in 20µL reagent from the RevertAid™ H Minus First Strand cDNA synthesis kit (Fermentas, Helsingborg, Sw) according to manual. Reverse transcriptase (RT)-polymerease chain reaction (PCR) carried out with 1µL cDNA using the following primers: GAPDH forward 5' AACGACCCCTTCATTGAC, 3', reverse 5' TCCACGACATACTCAGCAC 3', Ferroportin forward 5' CCCTGCTCTGGCTGTAAAAG 3', reverse 5' AACAAGGCCACATTTTCGAC 3', Hepcidin forward 5' GCTTCTCCTCCTGGCCAGCCTGAGC 3', reverse 5' CGCTTCAGCATCAGCAGCGCACTGT 3' and DreamTaq™ Green PCR Master Mix (Fermentas, Helsingborg, Sw). The Veriti 96 Well Thermal cycler (Applied Biosystems, Naerum, DK) was used with the following settings: 1x: 95°C 1 min.; 30x: 95°C 15 s.; 60°C 15 s.; 72°C 15 s.; 1x: 72°C 7 min. The control reaction was performed identically except that 1 µL total RNA was used instead of cDNA as a template.
Subsequently, the samples were run on a 2% agarose gel, using a Wide Mini Sub Cell (Biorad) with a Power Supply Model 250/2,5 (Biorad). The gel was processed on a Kodak Image Station 4000 mm Pro (Carestream Health, USA) with an excitation filter of 530 nm and an emission filter of 600 nm.

Quantitative RT-PCR (qRT-PCR) analyses of brain, liver and duodenum samples were performed using the Stratagene Mx 3000 system. Duplex samples were run with the following program: 95°C for 10 minnuttes, followed by 40 cycles of: 95°C for 30 sec. and 60°C for 15 sec. ending with a dissociation program.
The GAPDH, ferroportin and hepcidin primers were the same as used for RT-PCR and as a control for normalization the GAPDH primers were utilized. The duodenal ferroportin/GAPDH ratio and the liver hepcidin/GAPDH ratio was considered 100%. The ferroportin/GAPDH ratio and hepcidin/GAPDH ratio was expressed relatively to the duodenum and liver respectively.

2.4.2 TOTAL IRON CONTENT

Inductively coupled plasma mass spectrometry (ICP-MS) was used to determine the total amount of iron (µg Fe/g brain tissue) in the groups. The cortex cerebri from the ID (n=5), ID + Fe (n=5) and control (n=5) was used, as well as the cerebellum (n=5), the VTA (n=3-5) and the habenula (n=5) of the 2 week, 8 week and 8 month old rats. The tissue was weighed and thereafter placed in MF100 vessels (Anton Paar, Graz, AT) along with 2 ml MilliQ H_2O, 2 ml 67,5% HNO_3 and 200 µm 10 ppm yttrium (1 ml yttrium, 20 ml 67,5% HNO_3 and 79 ml MilliQ H_2O). The MF100 vessels were then microwaved in a Multiwave 3000 (Anton Paar, Graz, AT) in 3 stages: Warming up to 1200 watt for 10 minutes, warming for 10 minutes at 1200 watt and a cool-down stage which lasted 15 minutes. The tissue was transferred to measuring tubes, filled to 10 ml with MilliQ H_2O, and subsequently transferred to tubes used for ICP-MS.
A metal standard consisting of Cu, Zn and Fe solutions of 1000 ppb, 100 ppb and 10 ppb respectively, was prepared by adding 5 ml, 0,5 ml and 0,05 ml of a 10000 ppb stock solution (0,5 ml Cu, 0,5 ml Zn, 0,5 ml Fe and 10 ml HNO_3 and 38,5 ml MilliQ H_2O) to 10ml HNO_3 and filling up to 50 ml with MilliQ H_2O. ICP-MS was carried out on all tissue samples for approximately 45 minutes with an ICAP 6000

series (Thermo Scientific, Slangerup, Denmark) and the results were obtained from iTEVA ICP software (Thermo Scientific, Slangerup, Denmark).

2.5 STATISTICS

The software application PASW Statistics 18.0 (SPSS Inc., Chicago, USA) was used for statistical analysis along with Microsoft Excel 2010 (Microsoft, Redmond, USA) and GraphPad Prism 5,0 (GraphPad Software, California, USA).
SPSS 17.0 and GraphPad prism was used to calculate means, significant differences, standard error of mean (SEM) and produce statistical illustrations. Microsoft Excel 2010 was mainly used for organizing data from trial protocols. These files were then converted into a file type (Comma-Separated-Values files) supported by SPSS 17.0 and GraphPad Prism.
The samples were examined for Normal distribution. This was done by an evaluation of histograms, Q-Q plots and performing Shapiro–Wilk test for normality. If data were Normal distributed a One-way ANOVA was used to compare more than two populations. Non-parametric tests were used if data was not Normal distributed.
The significance level in this study was set to $\alpha=0.05$ ($p=0.05$), meaning that a difference is significant when the probability of seeing no difference is lower than 5%.

3 RESULTS

3.1 DEVELOPING RATS

3.1.1 TOTAL IRON CONTENT

Total iron measurements revealed an increase in iron content with aging. The analysis was conducted on preparations of cerebellum, VTA and habenula from 2 week, 8 week and 8 month old rats. The mean iron amount is illustrated in Figure 4 and the exact mean is listed in Table 4. The lowest amount of iron was discovered in the 2 week old rats in every brain region.
8 week old rats had an increased amount of iron, compared to the 2 week old rats and the highest amount was detected in the 8 month old rats. Regional differences existed as well, with the highest amounts of iron in the cerebellum and the lowest content in the habenula.
Statistical comparisons between the brain regions and ages showed that the iron amount of the 2 week old rats was significantly ($p < 0.05$) lower compared to both 8 week and 8 month old rats. Furthermore, a significant difference was observed between the 8 week old and 8 month old rats in the habenula region.

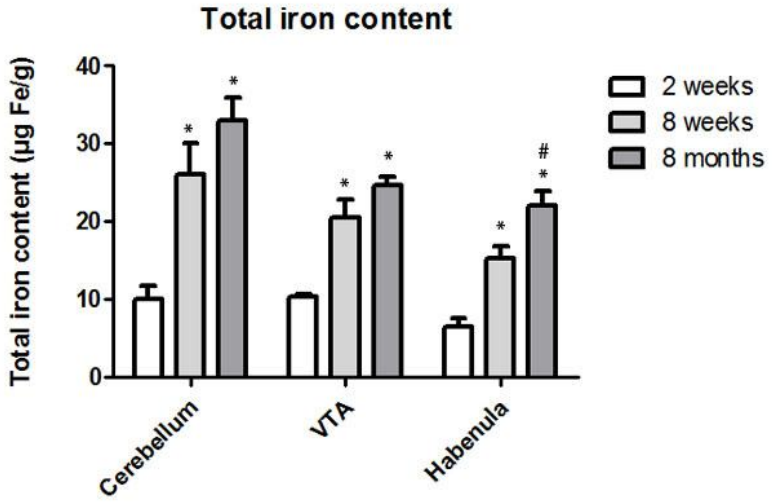

Figure 4: Total iron content (µg Fe/g) of microdissected preparations of cerebellum, VTA and habenula from 2 week, 8 week and 8 month old rats. Data are expressed as means ± SEM. Asterisks (*) indicate a significant difference from the 2 weeks old rats ($p < 0.05$) and number sign (#) indicate a significant difference from the 8 weeks old rats ($p < 0.05$).

Table 4: Total iron content in the cerebellum, VTA and habenula of different aged rats. The mean value is stated with± SEM.

Age	Total iron in cerebellum (µg Fe/g)	Total iron in VTA (µg Fe/g)	Total iron in habenula (µg Fe/g)
2 weeks	9.96 ±1.71 (n=5)	10.26 ±0.29 (n=5)	6.45 ±1.09 (n=4)
8 weeks	26.07 ±4.01 (n=5)	20.53 ±2.30 (n=3)	15,30 ±1.50 (n=5)
8 months	32.95 ±2.98 (n=5)	24.56 ±1.10 (n=5)	21.98 ±1.86 (n=5)

3.1.2 EXPRESSION OF FERROPORTIN MRNA

Quantitative RT-PCR was used to determine the mRNA expression of ferroportin in microdissected preparations of cerebellum, VTA and habenula in different aged rats. Expression of ferroportin is presented in Figure 5a as relative quantity normalized to GAPDH and correlated to the expression of ferroportin in the duodenum of normal aged rats (8 weeks), which was set to 100%. Figure 5b illustrates a representative gel of the RT-PCR product of ferroportin and GAPDH in the VTA region of different aged rats. The exact means are listed in
Table 5.
Figure 5 clearly demonstrates a decrease in ferroportin mRNA with aging. It is seen visually in the ethidium bromide gel illustration and in the more sensitive RT-qPCR analysis where the difference was measurable. A significant difference ($p < 0.05$) was observed in the 2 week old rats compared to the 8 week and 8 month old rats in all brain areas, except in the habenula region where a significant difference ($p < 0.05$) was detected in the 2 week old rats versus the 8 month old rats.

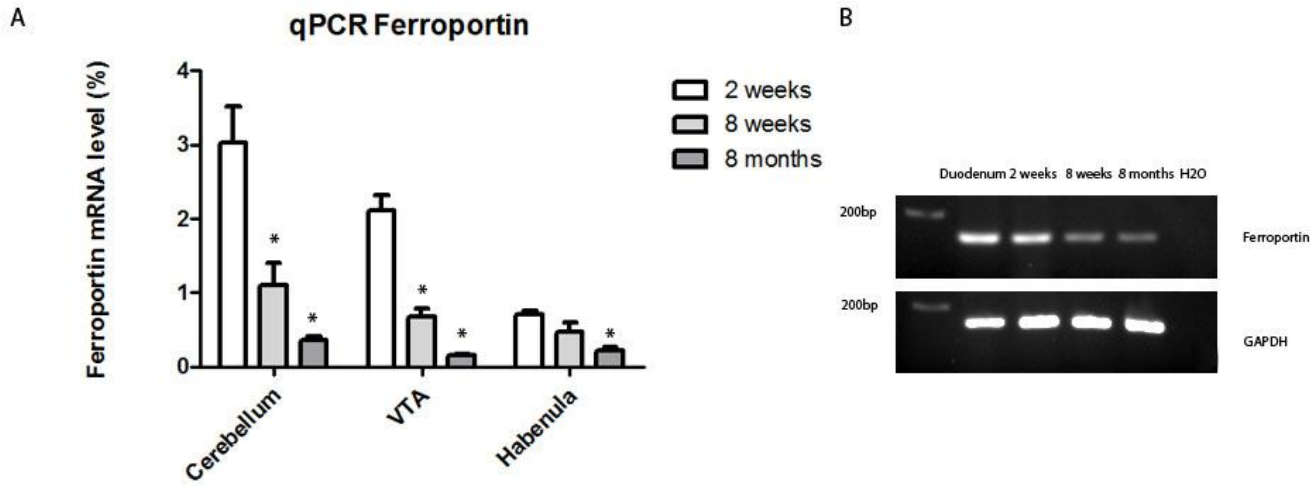

Figure 5: Brain ferroportin gene expression in rats of different age. ***a*** *RT-qPCR analysis of microdissected preparations of cerebellum, VTA and habenula from 2 week, 8 week and 8 month old rats. The expression of ferroportin, normalized with GAPDH, in the different brain regions was correlated to the ferroportin expression in the duodenum, which was arbitrarily set to 100%. Data are expressed as means ± SEM. Asterisks (*) indicate a significant difference from the 2 weeks old rats ($p < 0.05$).* ***b*** *A representative ethidium bromide stained gel showing the RT-PCR product of ferroportin and GAPDH in the VTA region. Lane 1: 100bp ladder molecule weight marker (generuler). Lane 2: microdissected duodenum of normal aged rats. Lane 3-5: microdissected VTA of 2 week, 8 week and 8 month old rats. Lane 6: H_2O.*

Table 5: Ferroportin mRNA expression in the cerebellum. VTA and habenula of different aged rats. The mean value is stated with± SEM.

Age	Ferroportin mRNA level in cerebellum (%)	Ferroportin mRNA level in VTA (%)	Ferroportin mRNA level in habenula (%)
2 weeks	3.03 ±0.45 (n=4)	2.11 ±0.21 (n=4)	0,71 ±0.05 (n=4)
8 weeks	1.11 ±0.29 (n=4)	0.68 ±0.10 (n=4)	0,48 ±0.12 (n=4)
8 months	0.37 ±0.04 (n=4)	0.16 ±0.05 (n=4)	0.22 ±0.05 (n=4)

3.1.3 EXPRESSION OF HEPCIDIN MRNA

Quantitative RT-PCR was used to determine the mRNA expression of hepcidin in microdissected preparations of cerebellum, VTA and habenula in different aged rats. Expression of hepcidin is presented in Figure 6a as relative quantity normalized to GAPDH and correlated to the expression of hepcidin in the liver of normal aged rats (8 weeks), which was set to 100%. Figure 6b illustrates a representative gel of the RT-PCR product of ferroportin and GAPDH in the VTA region of different aged rats. The exact means are listed in
Table 6.
Figure 6 shows that the brain was almost absent of hepcidin. There were no visible bands on the ethidium bromide gel regardless of age. The quantitative RT-PCR analysis revealed the presence of hepcidin in the different brain areas, albeit very low. No significant difference was detected between the groups.

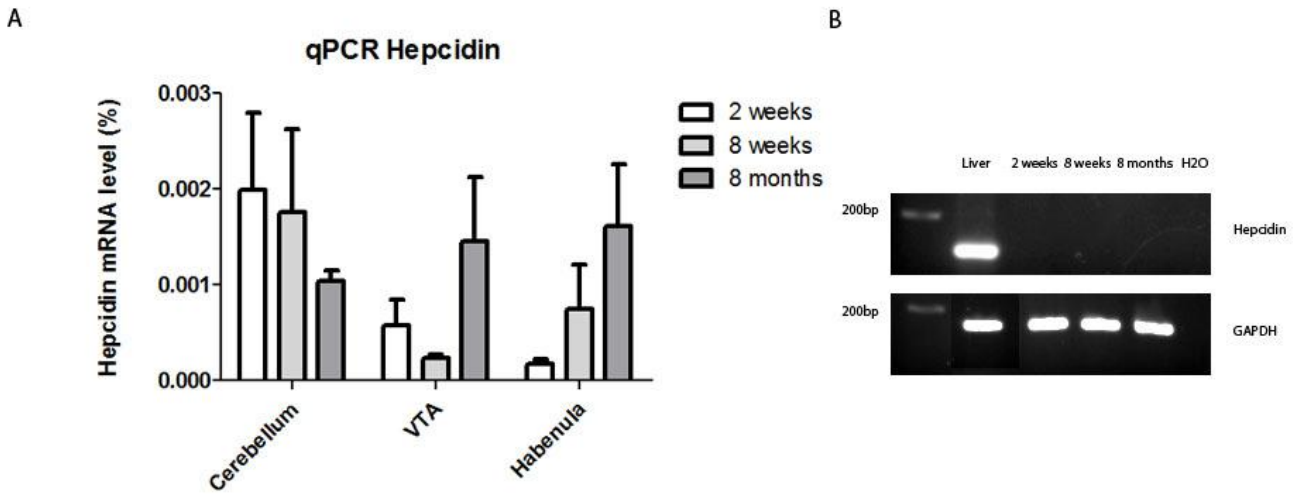

Figure 6: Brain hepcidin gene expression in rats of different age. A RT-qPCR analysis of microdissected preparations of cerebellum, VTA and habenula from 2 week, 8 week and 8 month old rats. ***a*** *The expression of hepcidin, normalized with GAPDH, in the different brain regions was correlated to the normalized hepcidin expression in the liver, which was arbitrarily set to 100%. Data are expressed as means ± standard error.* ***b*** *A representative ethidium bromide stained gel showing the PCR of product hepcidin and GAPDH in the VTA region. Lane 1: 100bp ladder molecule weight marker (generuler). Lane 2: microdissected duodenum of normal aged rats. Lane 3-5: microdissected VTA of 2 week, 8 week and 8 month old rats. Lane 6:* H_2O.

Table 6: Hepcidin mRNA expression in the cerebellum. VTA and habenula of different aged rats. The mean value is stated with± SEM.

Age	Hepcidin mRNA level in cerebellum (%)	Hepcidin mRNA level in VTA (%)	Hepcidin mRNA level in habenula (%)
2 weeks	0.002 ±0.0008 (n=4)	0.001 ±0.0003 (n=4)	0.0002 ±0.00004 (n=4)
8 weeks	0.002 ±0.0009 (n=4)	0.0002 ±0.00004 (n=4)	0.001 ±0.0005 (n=4)
8 months	0.001 ±0.0001 (n=4)	0.001 ±0.0007 (n=4)	0.002 ±0.0007 (n=4)

3.2 IRON STATUS RATS

3.2.1 TOTAL IRON CONTENT

Iron deficiency affects total iron measured in the cerebral cortex. The analysis was conducted on preparations of the cerebral cortex of control, ID and ID+Fe rats. The mean iron amount is illustrated in both Figure 7 and
Table 7.
The lowest amount of iron was discovered in the ID rats and the highest amount was seen in the control rats. There was no significant difference between the groups.

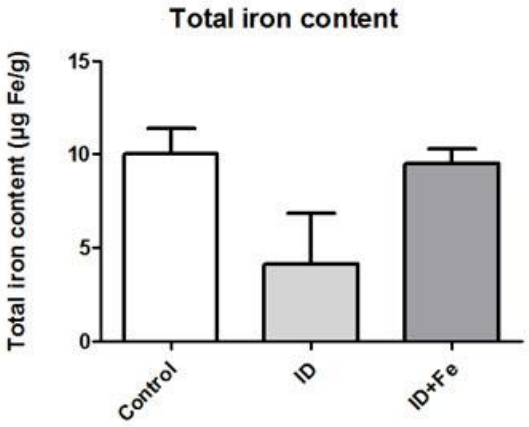

Figure 7: Total iron content (µg Fe/g) of microdissected preparations of the cerebral cortex of control, ID and ID+Fe rats. Data are expressed as means ± SEM.

Table 7: Total iron content in cerebral cortex of rats with different iron status. The mean value is stated with± SEM.

Group	Total iron in cortex (µg Fe/g)
Control	10.04 ±1.314 (n=4)
ID	4.110 ±2.719 (n=3)
ID + Fe	9.492 ±0.791 (n=4)

3.2.2 EXPRESSION OF FERROPORTIN MRNA

Quantitative RT-PCR was used to determine the mRNA expression of ferroportin in microdissected preparations of duodenum, liver, cerebral cortex, cerebellum, striatum and brainstem from control, ID and ID+Fe rats. Figure 8 displays the relative quantity of ferroportin mRNA normalized to GAPDH in the duodenum and liver and the exact values are listed in
Table 8. In the duodenum, the lowest amount of ferroportin mRNA was detected in the ID+Fe group and the highest amount in the ID group. Furthermore, a significant difference ($p < 0.05$) was found between the ID+Fe rats and the ID rats.
For the liver samples, the lowest amount of ferroportin mRNA was detected in the ID group and the highest amount in the ID+Fe group. Moreover, a significant difference ($p < 0.05$) existed when comparing the ID+Fe rats with the control and the ID rats.

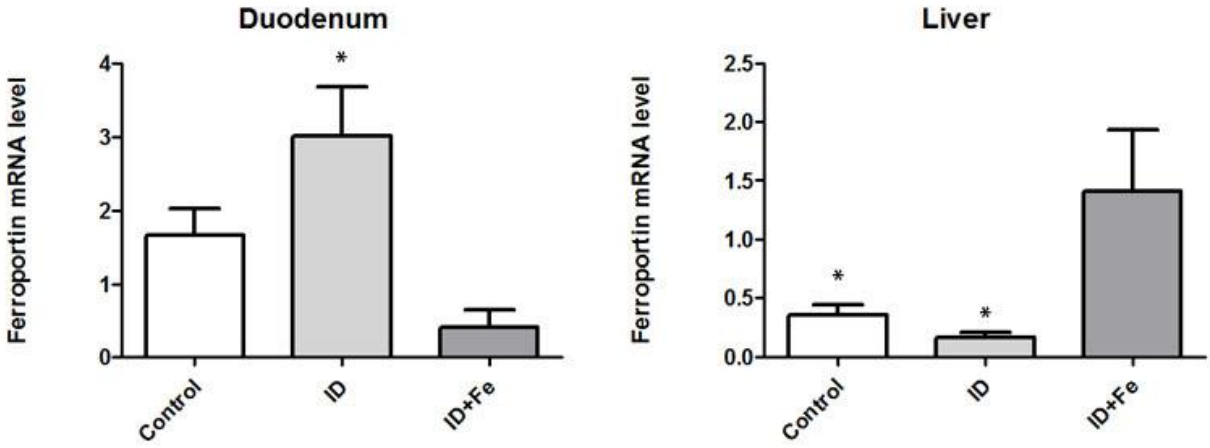

Figure 8: RT-qPCR ferroportin analysis of microdissected preparations of duodenum and liver from control, ID and ID+Fe rats. The relative expression of ferroportin is normalized with GAPDH and expressed as means ± SEM. Asterisks (*) indicate a significant difference from the ID+Fe rats ($p < 0.05$).

Table 8: Ferroportin mRNA expression in the liver and duodenum of rats with different iron status. The mean value is stated with± SEM

Group	Ferroportin mRNA level in duodenum	Ferroportin mRNA level In liver
Control	1.45 ±0.506 (n=4)	0.41 ±0.086 (n=5)
ID	3.94 ±0.880 (n=4)	0.52 ±0.320 (n=6)
ID + Fe	1.08 ±0.616 (n=3)	1.41 ±0.520 (n=3)

The expression of ferroportin in the brain is presented in Figure 9a as relative quantity normalized to GAPDH and correlated to the expression of ferroportin in the duodenum of normal aged rats (8 weeks), which was set to 100%.
Figure 9b illustrates a representative gel of the RT-PCR product of ferroportin and GAPDH in the cerebral cortex region of rats with different iron status. The exact means are listed in Table 9.
Figure 9 shows that the amount of ferroportin was low in all brain areas and groups. There was no significant difference between groups in any of the brain regions. However, a tendency existed in the cortex and the cerebellum where the highest amount of ferroportin was found in the ID+Fe rats and

the lowest amount in the control group. In the striatum and brain stem, the highest level of mRNA was detected in the control group and the lowest amount in the ID group.

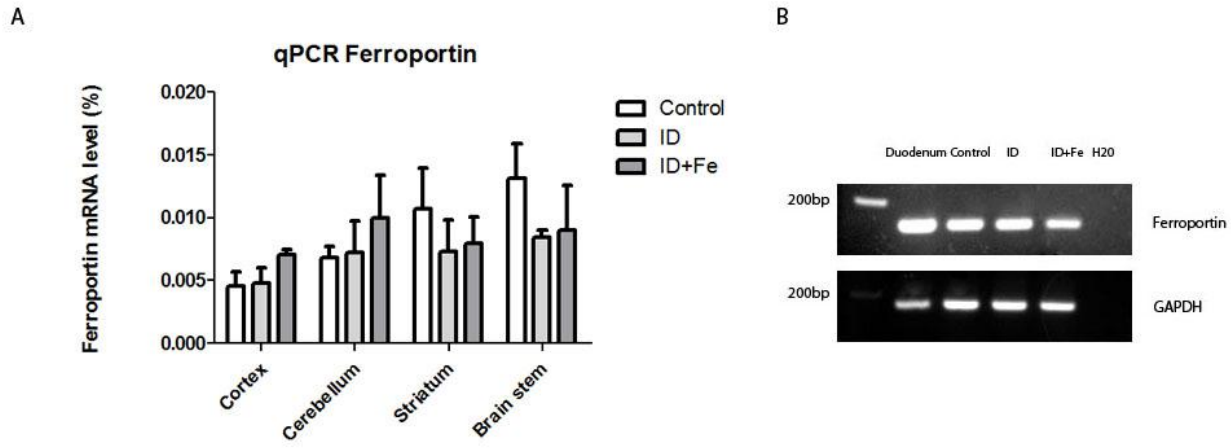

Figure 9: Brain ferroportin gene expression in rats of different iron status. ***a*** *RT-qPCR analysis of microdissected preparations of cortex, cerebellum, striatum and brainstem from control, ID and ID+Fe rats. The expression of ferroportin, normalized with GAPDH, in the different brain regions was correlated to the normalized ferroportin expression in the duodenum, which was arbitrarily set to 100%. %. Data are expressed as means ± standard error.* ***b*** *A representative ethidium bromide stained gel showing the RT-PCR product ferroportin and GAPDH in the cortex region. Lane 1: 100bp ladder molecule weight marker (generuler). Lane 2: microdissected duodenum of normal aged rats. Lane 3-5: microdissected cerebral cortex of Control, ID and ID+Fe rats. Lane 6: H_2O.*

Table 9: Ferroportin mRNA expression in the cortex, cerebellum, striatum and brain stem of of rats with different iron status. The mean value is stated with± SEM.

Group	Ferroportin mRNA level in cortex (%)	Ferroportin mRNA level in cerebellum (%)	Ferroportin mRNA level in striatum (%)	Ferroportin mRNA level in brain stem (%)
Control	0.005 ±0.0012 (n=4)	0.007 ±0.0009 (n=3)	0.011 ±0.0033 (n=4)	0.013 ±0.0028 (n=4)
ID	0.005 ±0.001 (n=4)	0.007 ±0.002 (n=4)	0.007 ±0.003 (n=4)	0.008 ±0.0005 (n=3)
ID + Fe	0.007 ±0.0004 (n=4)	0.010 ±0.0035 (n=4)	0.008 ±0.0021 (n=3)	0,009 ±0,0035 (n=4)

3.2.3 EXPRESSION OF HEPCIDIN MRNA

Quantitative RT-PCR was used to determine the mRNA expression of hepcidin in microdissected preparations of duodenum, liver, cerebral cortex, cerebellum, striatum and brainstem from control, ID and ID+Fe rats. Figure 10 displays the relative quantity of hepcidin mRNA normalized to GAPDH in the duodenum and liver and the exact means are listed in Table 10.
In the duodenum, the lowest amount of hepcidin mRNA was detected in the ID group and the highest amount in the ID+Fe group. Furthermore, a significant difference ($p < 0.05$) was found when comparing the ID+Fe rats with the control and ID rats.
For the liver samples, the lowest amount of hepcidin mRNA was detected in the ID group and the highest amount in the ID+Fe group. A significant difference ($p < 0.05$) was found when comparing the ID rats with the control and ID+Fe rats.

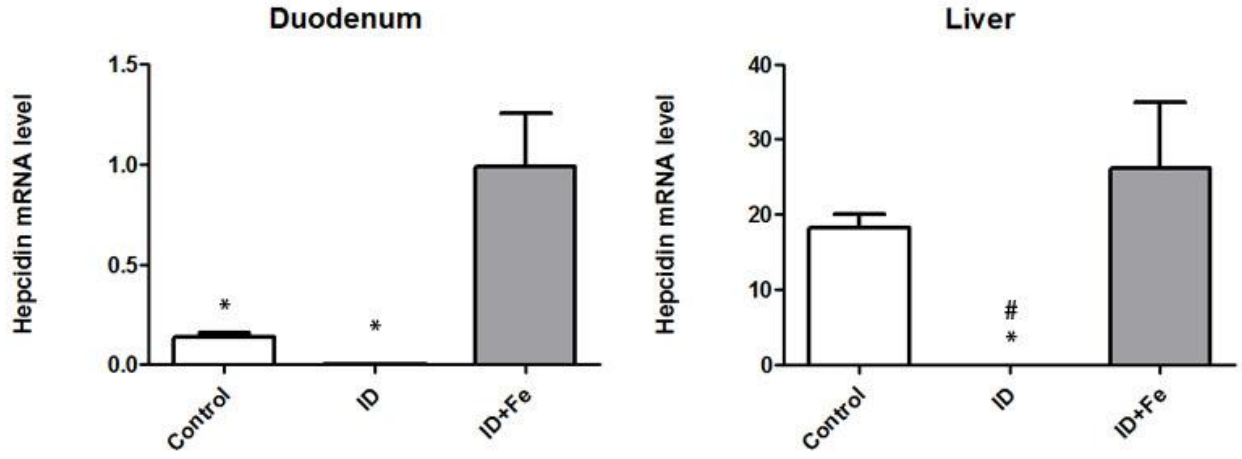

Figure 10: RT-qPCR hepcidin analysis of microdissected preparations of duodenum and liver from control, ID and ID+Fe rats. The expression of hepcidin is normalized with GAPDH and expressed as means ± standard error. Asterisks (*) indicate a significant difference from the ID+Fe rats ($p < 0.05$) and number sign (#) indicate a significant difference from the control rats ($p < 0.05$).

Table 10: Hepcidin mRNA expression in the liver and duodenum of rats with different iron status. The mean value is stated with± SEM.

Group	Hepcidin mRNA level in duodenum	Hepcidin mRNA level In liver
Control	0.139 ±0.019 (n=3)	18.22 ±1.783 (n=3)
ID	0.001 ±0.001 (n=5)	0.015 ±0.009 (n=6)
ID + Fe	0.987 ±0.268 (n=4)	26.19 ±8.679 (n=3)

The expression of hepcidin in the brain is presented in Figure 11a as relative quantity normalized to GAPDH and correlated to the expression of hepcidin in the duodenum of normal aged rats (8 weeks), which was set to 100%. Figure 11b illustrates a representative gel of the RT-PCR product of hepcidin

and GAPDH in the cerebral cortex region of rats with different iron status. The exact means are listed in Table 11.

The amount of hepcidin in the different brain areas was almost absent despite iron status. The ethidium bromide gel showed no hepcidin bands and the quantitative RT-PCR analysis revealed a very low detection of hepcidin.

There was no significant difference between groups in the cortex, cerebellum and striatum. A significant difference was found in the brain stem, when comparing the control rats with the ID and ID+Fe rats.

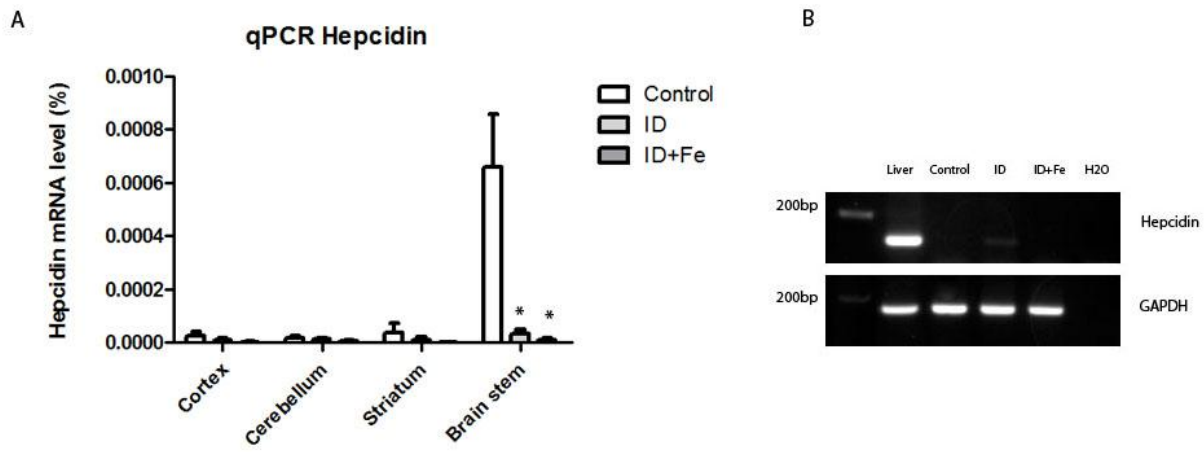

Figure 11: Brain hepcidin gene expression in rats of different iron status. ***a*** *RT-qPCR analysis of microdissected preparations of cortex, cerebellum, striatum and brainstem from control, ID and ID+Fe rats. The expression of hepcidin, normalized with GAPDH, in the different brain regions was correlated to the normalized hepcidin expression in the liver, which was arbitrarily set to 100%. %. Data are expressed as means ± standard error. Asterisks (*) indicate a significant difference from the control rats ($p < 0.05$).* ***b*** *A representative ethidium bromide stained gel showing the RT-PCR product of hepcidin and GAPDH in the cortex region. Lane 1: 100bp ladder molecule weight marker (generuler). Lane 2: microdissected duodenum of normal aged rats. Lane 3-5: microdissected cerebral cortex of Control, ID and ID+Fe rats. Lane 6: H_2O.*

Table 11: Hepcidin mRNA expression in the cortex, cerebellum, striatum and brain stem of of rats with different iron status. The mean value is stated with± SEM.

Group	Hepcidin mRNA level in cortex (%)	Hepcidin mRNA level in cerebellum (%)	Hepcidin mRNA level in striatum (%)	Hepcidin mRNA level in brain stem (%)
Control	$2.4*10^{-5}$ ±$1.8*10^{-5}$ (n=4)	$1.8*10^{-5}$ ±$0.7*10^{-5}$ (n=4)	$3.7*10^{-5}$ ±$3.5*10^{-5}$ (n=4)	$6.6*10^{-4}$ ±$2.0*10^{-4}$ (n=4)
ID	$1.1*10^{-5}$ ±$0.8*10^{-5}$ (n=4)	$1.3*10^{-5}$ ±$0.6*10^{-5}$ (n=4)	$1.1*10^{-5}$ ±$0.9*10^{-5}$ (n=4)	$3.2*10^{-5}$ ±$1.7*10^{-5}$ (n=4)
ID + Fe	$0.3*10^{-5}$ ±$0.1*10^{-5}$ (n=4)	$0.6*10^{-5}$ ±$0.2*10^{-5}$ (n=4)	$0.1*10^{-5}$ ±$4.7*10^{-7}$ (n=3)	$1.1*10^{-5}$ ±$0,8*10^{-5}$ (n=4)

4 DISCUSSION

4.1 EFFECT OF DEVELOPMENT ON TOTAL IRON CONTENT, FERROPORTIN AND HEPCIDIN GENE EXPRESSION

The results obtained in the present study showed that development has a significant effect on ferroportin gene expression as well as iron content in the brain. Whereas the effect and expression of hepcidin mRNA was limited.

Iron concentrations in various brain regions of different aged rats were similar to those reported by other investigators (Chang et al., 2005, Pinero et al., 2000). In all three regions, iron increased with age and the highest concentration was observed in the cerebellum.

Iron increase and age has been associated with oxidative stress and several neurodegenerative disorders, with Parkinson's disease and Alzheimer's disease as the most studied (Stankiewicz and Brass, 2009). Increasing iron deposits in the brain implies that the expression of iron transporters might be altered to avoid detrimental effects of oxidative stess. Molecules involved in iron metabolism is regulated both transcriptionally and post transcriptionally via feedback mechanisms in response to cellular iron status and other stimuli, such as hypoxia, nitric oxide, and oxidative stress (Chua et al., 2007). In the present study, development significantly changed brain iron levels, suggesting for a regulation in gene expression of iron transporters.

Previous studies have reported changes in ferroportin expression in the brain during rat development (Jiang et al., 2002, Moos and Rosengren Nielsen, 2006). This was confirmed in the present study where ferroportin gene expression was significantly altered by age in the cerebellum, VTA and habenula area. Surprisingly, the mRNA expression of ferroportin declined with age despite an increase in iron. This was unexpected, since semi quantitative western blot analysis of the ferroportin protein expression, showed an increase with age in male Sprague-Dawley rats (Jiang et al., 2002). The decline in ferroportin mRNA could be attributed to a similar mechanism of transcriptional regulation observed in enterocytes of the duodenum, where an alternative ferroportin transcript, ferroportin1B, has been identified (Zhang et al., 2009). This implies that there might be another form of ferroportin expression in the brain, and that ferroportin in the brain is regulated on the transcriptional and translational level. Further research is needed to confirm the presence of another ferroportin transcript.

Ferroportin is down regulated post-transnationally by the liver-produced peptide hepcidin. The hepcidin-ferroportin interaction causes the ferroportin protein to degrade and redistribute from the cellular membrane to the cytoplasm (Nemeth et al., 2004).

Recent studies have identified hepcidin gene and protein expression in the murine brain, including the cerebral cortex, hippocampus, amygdala, thalamus, hypothalamus, mesencephalon, cerebellum and pons (Wang et al., 2010, Zechel et al., 2006). Moreover, hepcidin has been found in both neurons and glia cells (Zechel et al., 2006). Our data confirms the presence of hepcidin mRNA in the rodent brain, however to a minimal extent. The hepcidin mRNA expression was not visible on ethidium stained gels and the quantification by qRT-PCR revealed an expression of 0.0002% – 0.00002% compared to the liver hepcidin expression. Supporting this observation, Zechel and colleagues were unable to detect hepcidin mRNA expression by in situ hybridization, probably because of low mRNA signal strength (Zechel et al., 2006).

The gene expression of hepcidin has been shown to increase with age in the mouse brain (Wang et al., 2010), suggesting a regulation pattern similar to other tissues, where an increase in iron leads to an increase in hepcidin (Pigeon et al., 2001). Contradicting these observations, the results in this study showed no significant change in hepcidin mRNA expression in the rat brain. However, a trend of increased hepcidin with age was detected in the habenula region. It is possible that inter-species variation between rat and mouse, might account for the inconclusive results found in hepcidin mRNA level during development.

While hepcidin is well known to have a pivotal role in peripheral iron metabolism, the function of hepcidin in the brain is not fully understood. Results in the present study and earlier research indicate, that hepcidin might be central in the regulation of brain iron efflux. The importance of hepcidin has been shown in a recent study. Wang and colleagues, found that conditional Smad4 knockout mice display a dramatic reduction in hepcidin levels as well as an iron overload in the liver, pancreas, kidney, and to some extent the brain (Wang et al., 2005). Furthermore, intracerebroventricular injection of hepcidin modulates the expression of ceruloplasmin, DMT1 and ferroportin in the rat cerebral cortex and hippocampus. Misregulation of hepcidin has also been connected with neurodegenerative processes (Zechel et al., 2006).

Even though hepcidin seems to be needed in brain iron metabolism, the exact molecular mechanism, the regulation and the linkage between hepcidin and other iron regulators in the brain is still unknown.

4.2 Effect of iron status on total iron content, ferroportin and hepcidin gene expression

It has been well documented that dietary iron severely affects iron content, ferroportin and hepcidin expression in the liver and duodenum (Abboud and Haile, 2000, Kim et al., 2007, Mazur et al., 2003). The present study confirms that ferroportin and hepcidin gene expression is significantly altered in rats of different iron status. Ferroportin mRNA level is significantly up regulated in the duodenum of iron deficient rats, compared to rats receiving iron supplements. This is in accordance with other studies and concludes that ferroportin mRNA is regulated via an alternative transcript (Abboud and Haile, 2000, Zhang et al., 2009). In the liver, ferroportin mRNA expression is significantly higher in rats receiving iron supplements, compared to control and iron deficient rats. This clearly demonstrates that ferroportin in the liver is regulated through the IRE-IRP pathway, and it parallels the expression pattern of ferritin (Abboud and Haile, 2000).

Iron metabolism, including ferroportin and hepcidin, in the brain have been less investigated, even though iron deficiency during early development significantly alters brain iron stores (Beard et al., 2003, Jorgenson et al., 2003).

The analysis of cerebral cortex total iron content in the present study between control, iron deficient and iron reverted rats, revealed that dietary iron status have an effect on brain iron content, although not significant. Previous investigations (unpublished) have shown, that iron deficiency during early development significantly decreases total iron content in the male rat brain, in line with other studies (Beard et al., 2003, Jorgenson et al., 2003). The fact that no significant difference was found between iron deficient rats given iron supplement and control rats, suggests that brain iron content can be completely reverted with early administration of iron supplementation. Altogether, it can be concluded that systemic iron deficiency has a great impact on brain iron content, and thus it would be antici-

pated that constituents involved with iron metabolism might be changed. This is, however, not the case in the present study where no significant alteration in ferroportin gene expression was observed in different brain areas of iron deficient, iron reverted and control rats.
Hypothetically, ample iron would increase the expression of ferroportin and iron deficiency would decrease the expression. A tendency of this statement was observed in the present study, albeit not significantly.
Other investigators have also looked at ferroportin expression in animal models with altered brain iron transport. DMT1 mutated Belgrade rats suffer from an insufficient iron uptake by the brain and exhibit unaltered levels of ferroportin (Burdo et al., 2001). Moreover, Wu and colleagues studied mice with a mutated IRP2, which leads to neurodegeneration and regional increases in brain iron, but found no changes in the expression of ferroportin (Wu et al., 2004). The results of these studies, including the fact that age seems to have a significant effect on ferroportin gene expression, implies that transcriptional regulation of ferroportin is complex and cannot be attributed to iron status alone. Additional research is needed, especially on the cellular level, in order to discover the regulative mechanisms behind iron transport in the brain.

Hepcidin, the small liver produced peptide, is extremely sensitive to changes in iron status. This has been demonstrated in several studies (Mazur et al., 2003, Nicolas et al., 2002) and was confirmed in the present investigation. We showed a significant higher level of hepcidin mRNA expression in the duodenum of rats receiving iron supplements compared to iron deficient rats. The same expression pattern applied in the liver, where iron deficient rats almost were devoid of hepcidin mRNA.
The expression of hepcidin in the brain is a relatively new research area, with many unanswered questions. Wang et al. (2010) speculate that hepcidin brain expression could be responsive to stimuli similar of those found in the peripheral organs (Wang et al., 2010), whereas other groups have suggested that hepcidin plays a distinct and independent role in brain iron metabolism (Zechel et al., 2006).
Our results indicate that regulation of hepcidin mRNA expression is different in the brain, compared to the liver and duodenum. The hepcidin gene was expressed at extremely low levels in all brain areas investigated, despite differences in brain iron levels and nutritional status. Interestingly, it seems that the highest hepcidin amount was found in control rats and the lowest amount in the iron reverted rats. This implies that iron status in the brain, is a minor regulation parameter and that other factors most be of higher importance, when considering hepcidin gene regulation. It could be enlightening to look at the expression of hepcidin in animal models with compromised blood-brain barrier integrity. Hypothetically, pathological brain conditions would be accompanied by migration of inflammatory cells into the brain, that would have a great influence on both hepcidin and ferroportin expression.

5 CONCLUSION

This thesis studied the effect of iron status and development on ferroportin and hepcidin gene expression in the rat brain. Analyzed microdissected brain samples of different aged rats, revealed that aging significantly increased brain iron concentrations, with the highest amount in the cerebellum. Moreover, ferroportin gene expression was significantly altered by age in the cerebellum, VTA and habenula area. Surprisingly, the mRNA expression of ferroportin declined with age despite an increase in iron, which was unexpected.

The present study confirmed the presence of hepcidin mRNA in the rat brain, however to a minimal extent. Furthermore, age had no significant effect on hepcidin gene expression in the rat brain.

Iron status was shown to have a highly significant effect on hepcidin and ferroportin in the liver and duodenum. Ferroportin mRNA level was significantly up regulated in the duodenum of iron deficient rats compared to rats receiving iron supplements and vice versa in the liver.

The analysis of cerebral cortex total iron content in the present study between control, iron deficient and iron reverted rats, revealed that dietary iron status has an effect on brain iron content, although not significant. This is probably due to a low number of brain samples analyzed.

No significant alteration in ferroportin gene expression was observed in different brain areas of iron deficient, iron reverted and control rats.

The level of hepcidin mRNA expression in the liver and duodenum, of rats receiving iron supplements compared to iron deficient rats, was significantly higher. Moreover, the results indicate that regulation of hepcidin mRNA expression is different in the brain, compared to the liver and duodenum. The hepcidin gene was expressed at extremely low levels in all brain areas investigated, despite differences in brain iron level and nutritional status

In conclusion, iron status and development does seem to have some effects on ferroportin and hepcidin gene expression, and apparently the age induced brain iron increase alone is not enough to alter transport mechanisms. This implies that other factors factors during development might influence the expression of key iron transport molecules in the brain.

6 ABBREVIATIONS

5-HT: Serotonin
BBB: Blood brain barrier
BCEC: Brain capillary endothelial cells
BDNF: Brain derived neurotrophic factor
BMP6: Bone morphogenic protein 6
CNPase: Cyclic nucleotide 3´-phosphohydrolase
CNS: Central nervous system
DA: Dopamine
DAT: Dopamine transporter
Dcytb: Duodenal cytochrome b
DMT1: Divalent metal transporter 1
EGF: Epithelial growth factor
Fe^{3+}: Ferric iron
Fe^{2+}: Ferrous iron
Fpn: Ferroportin
GDNF: Glia-derived neurotrophic factor
HAMP: human hepcidin gene
HCP1: Heme carrier protein
HEPH: Hephaestin
HJV: hemojuvelin
HO-1: Heme oxygenase 1
ICP-MS: Inductively Coupled Plasma Mass Spectrometry
ID: Iron deficient
ID+Fe: Iron deficient rats given isomaltoside 1000 injections
IRE: iron-responsive element
IRP: Iron-responsive protein
MBP: Myelin basic protein
NA: Noradrenalin
NGF: Nerve growth factor
P: Post natal day
qRT- PCR: Quantitative reverse transcriptase-polymerase chain reaction
RT-PCR: Reverse transcriptase-polymerase chain reaction
SEM: Standard error of mean
Tf: Transferrin
Tf-Fe: Transferrin bound iron
TfR: Transferrin receptor
TfR1: Transferrin receptor 1
VTA: Ventral tegmental area
UTR: Untranslated region

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