Thesis

Brain iron and cognition in healthy aging - Kognition bei Eisenablagerung im Hirn während des gesunden Alterns

submitted by

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research of this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of "Good Scientific Practice Date 26.01.2015".

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Abbreviations

αCTF	Alpha-Carboxy Terminal Fragment
AD	Alzheimer's Disease
AICD	AβPP Intracellular Domain
ALS	Amyotrophic Lateral Sclerosis
APOEε4	Apolipoprotein Eε4
ASPS	Austrian Stroke Prevention Study
ASPS-Fam	Austrian Stroke Prevention Family Study
ATP	Adenosine Triphosphate
Aβ	Amyloid-β Peptide
AβPP	Amyloid- β Protein Precursor
BBB	Blood Brain Barrier
BCEC	Blood Capillary Endothelial Cell
BL1	Basal Lamina 1
BL2	Basal Lamina 2
BMP	Bone Morphogenetic Protein
CDR	Clinical Dementia Rating
CNPase	2',3'-Cyclic Nucleotide 3'-Phosphodiesterase
CNS	Central Nervous System
CPMG	Carr-Purcell-Meiboom-Gill
CSF	Cerebrospinal Fluid
DA	Dopaminergic
DCT1	Divalent Cation Transporter
Dcytb	Duodenal Cytochrome B
DLBD	Diffuse Lewy Bodies Disease
DMT1	Divalent Metal Transporter
DNA	Deoxyribonucleic Acid

EAE	Experimental Autoimmune Encephalomyelitis
FA	Friedreich's Ataxia
FDG-PET	Fludeoxyglucose-Positron Emission Tomography
FDR	False Discovery Rate
FDRI	Field Dependent Rate Increase
Fe_2–Tf	Diferric Transferrin
Fe^{3+}	Ferric Iron
FLASH	Fast Low Angle Shot
FSE	Fast Spin Echo
FTL	Ferritin Light Polypeptide
GESEPI	Gradient Echo Slice Excitation Profile Imaging
GESFIDE	Gradient Echo Sampling Of Free Induction Decay And Echo
GSH	Glutathione
GSSH	Oxidized Glutathione
HAMP	Hepcidin Antimicrobial Peptide
HD	Huntington's Disease
HF	H-Ferritin
HH	Hereditary Hemochromatosis
HMG-CoA	3-Hydroxy-3-Methyl-Glutaryl-CoA
HNE	4-Hydroxynonenal
HSS	Hallervorden-Spatz Disease
INAD	Infantile Neuroaxonal Dystrophy
iNOS	Nitric Oxide Synthase
IRE	Iron Regulatory Element
IRP	Iron Regulatory Protein
LFI	Local Field Inhomogeneities
LIP	Labile Iron Pool
MAO	Monoamine Oxidase

MCI	Mild Cognitive Impairment
MFC	Magnetic Field Correlation
MMSE	Mini Mental State Examination
MPRAGE	Magnetization Preparation And Rapid Gradient Echo
MPTP	1-Methyl-4-Phenyl-1,2,3,6-Tetrapyridine
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
MTL	Medial Temporal Lobe
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NBIA	Neurodegeneration With Brain Iron Accumulation
NFT	Neurofibrillary Tangles
NFκB	NFKappaB
NO	Nitric Oxide
Nramp2	Natural Resistance-Associated Macrophage Protein
NTBI	Non-Transferrin-Bound Iron
6-OHDA	6-Hydroxydopamine
PD	Parkinson’s Disease
PFC	Prefrontal Cortex
PKAN	Pantothenate Kinase-Associated Neurodegeneration
PLAN	PLA2G6-Associated Neurodegeneration
PRIME	Partially Refocused Interleaved Multiple Echo
PUFA	n-6 Polyunsaturated Fatty Acids
RF	Radiofrequency
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SDR2	Stromal Cell-Derived Receptor 2
SN	Substantia Nigra

SNP	Single Nucleotide Polymorphism
SNPC	Substantia Nigra Pars Compacta
Steap3	6-Transmembrane Epithelial Antigen Of The Prostate 3
SWI	Susceptibility Weighted Imaging
Tf	Transferrin
TfR	Transferrin Receptors
TIV	Total Intracranial Volume
WMH	White Matter Hyperintensities

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Zusammenfassung

Ziele: Es ist bekannt, dass sich Eisen während des Alterns im Hirn ablagert und dass diese Eisenablagerung mit diveresen neurodegenerativen Erkrankungen, einschließlich der Alzheimer Erkrankung, im Zusammenhang steht. Der Eisengehalt im Hirngewebe kann in vivo mittels Relaxometrie /R2*-Kartierung im MRT erfasst werden. Mit dieser Studie wollten wir untersuchen, ob während der normalen Hirnalterung ein hoher Eisengehalt mit regionenspezifischen Kognitionseinbußen zusammenhängt.

Methoden: Zu diesem Zweck wurden 336 gesunde Teilnehmer aus der Austrian Stroke Prevention Family Study (ASPS-Fam) zwischen 38 und 86 Jahren in die Studie eingeschlossen. Die MR-Bildgebung und R2*-Kartierung der Basalganglien und des Neokortex erfolgte mittels 3T Scanner. Eine umfassende neuropsychologische Testung bewertete folgende kognitive Leistungen: Gedächtnis, exekutive Funktionen und psychomotorische Geschwindigkeit. Die Beziehung zwischen R2*-basierenden Eisenwerten und domänenspezifischer kognitiver Testleistung wurden mittels angepasster gemischter Modelle berechnet. Um zu testen, ob vaskulärer Hirnläsionen oder Hirnatrophie mediierende Effekte auf die Beziehung zwischen Eisen und kognitive Leistung ausüben, wurden einfache Mediationsmodele zur Schätzung indirekter Effektgrößen verwendet.

Ergebnisse: Wir dokumentierten den höchsten Eisengehalt im Globus Pallidus. Das Eisen im Globus Pallidus als auch im Putamen beeinträchtigte signifikanterweise, bis auf die Gedächtnisleistung, alle kognitiven Kategorien. Diese Wechselbeziehung stand mit der Eisenmenge im Zusammenhang. Vaskuläre Hirnläsionen und Hirnvolumen übten keinen mediierender Effekt auf die Beziehung zwischen Eisen und kognitiver Leistung aus.

Schlussfolgerung: Daraus schließen wir, dass während des Alterns, unabhänging von begleitenden Hirnanomalien, der R2*-basierende Eisengehalt in den Basalganglien mit Kognitionseinbußen korreliert. Die prognostische Bedeutung dieser Ergebnisse muss noch bestimmt werden.

Abstract

Objective: Brain iron accumulates during aging and has been associated with neurodegenerative disorders including Alzheimer´s disease. MR-based R2* mapping enables the in vivo detection of iron content in brain tissue. We here investigated if during normal brain aging increasing iron load relates to cognitive impairment in region-specific patterns.

Methods: We included 336 healthy participants from the Austrian Stroke Prevention Family Study (ASPS-Fam) aged 38 to 86 years. MR imaging and R2* mapping in the basal ganglia and neocortex was done at 3T. Comprehensive neuropsychological testing assessed memory, executive function and psychomotor speed. The associations between R2*-based regional iron content and domain-specific neuropsychological test performance were assessed by adjusted mixed models. To test if iron effects on cognition were mediated by vascular brain lesions or brain atrophy we used simple mediation models for estimating indirect effect sizes.

Results: We found the highest iron concentration in the globus pallidus, and pallidal as well as putaminal iron was significantly and inversely associated with cognitive performance in all cognitive domains, except memory. These associations were iron load-dependent. Vascular brain lesions and brain volume did not mediate the relationship between iron and cognitive performance.

Conclusion: We conclude that higher R2*-determined iron in the basal ganglia correlate with cognitive impairment during brain aging independent of concomitant brain abnormalities. The prognostic significance of this finding needs to be determined.

1. INTRODUCTION

Iron plays a central role in many biological processes such as cellular aerobic metabolism, deoxyribonucleic acid (DNA), neurotransmitter and myelin synthesis. It plays an important role in electron transfer and is a cofactor for a large number of enzymes (Crichton 2008). On the other hand, iron is also involved in processes that are suspected to be injurious for neural and other cellular substrates, such as the participation in the generation of reactive oxygen species (ROS) (Crichton 2001, Schipper 2004, Zecca, Youdim et al. 2004a) and the aggregation of α-synuclein (Kostka, Hogen et al. 2008) in neurons.

Iron progressively accumulates in the central nervous system (CNS) as a function of aging and it is preferentially located in the basal ganglia, hippocampus, cerebellar nuclei, and subcortical brain regions (Hallgren, Sourander 1958).

Abnormal accumulation of iron has been reported in numerous neurodegenerative and inflammatory CNS disorders (Brass, Chen et al. 2006, Zecca, Youdim et al. 2004a). However, it is unclear if increased iron deposition contributes to the pathogenesis of these diseases or only represents an epiphenomenon.

Magnetic resonance imaging (MRI) is sensitive to the existence and concentration of non-heme iron in the living human brain. The presence of iron deposits in brain- and other tissues causes local magnetic field inhomogeneities with decreased T2 (transverse) relaxation time resulting in hypointense signals on T2-weighted and T2* images, particularly at higher field strengths (Peran, Hagberg et al. 2007, Stankiewicz, Panter et al. 2007, Schenck, Zimmerman 2004). A further MRI technique that recently has been confirmed in a post-mortem study (Langkammer, Krebs et al. 2010) to represent an indirect measure of iron, is mapping of the relaxation rate R2* (1/T2*). These techniques enable in vivo studies and offer the opportunity to correlate regional iron concentrations in brain tissue to clinical phenotypes.

If iron-related oxidative stress precedes the hallmark of neuropathological lesions of neurodegeneration, it is likely that regional iron concentrations in brain tissue correlate to clinical findings at the earliest stages of neurodegenerative disorders.

So far MRI has been mainly used to evaluate brain iron accumulation in multiple sclerosis (Khalil, Langkammer et al. 2011) and neurodegenerative diseases including Alzheimer's disease and Parkinson's disease (Bartzokis, Sultzer et al. 2000, Brass, Chen et al. 2006, Thomas, Boyko et al. 1993). Only few authors (Rodrigue, Daugherty et al. 2013, Pujol, Junque et al. 1992, Sullivan, Adalsteinsson et al. 2009, Bartzokis, Lu et al. 2011) have investigated the association of brain iron and cognition in non-demented individuals. A recent report has suggested an inverse relationship between iron deposits in the basal ganglia and cognition in the general population (Penke, Valdes Hernandez et al. 2012).
Therefore, the purpose of the present study was to investigate the impact of R2*-based brain iron on cognition in healthy individuals free of history or signs of stroke and dementia.

1.1. Iron chemistry and the biological role of iron

1.1.1. Iron chemistry

Iron is an essential metal for almost all living organism on earth, participating in plenty of biochemical processes. It is the 26th element in the periodic table, a d-block transition element that can exist in oxidation states ranging from -2 to +6. Only ferrous (+2) and ferric (+3) oxidation states appear commonly in biological systems (for a comprehensive review, see (Crichton 2001). Generally, the neutral iron atom has four unpaired electrons in the 3d orbital and two paired electrons in the 4s orbital. The 2+ oxidation state is generated by removing the 4s electrons, while further removal of one 3d electron generates the 3+ state (Sienko, Plane 1976). While Fe^{2+} is extremely water soluble, Fe^{3+} is relatively insoluble in water ($K_{sp} = 10^{-39}$ M and at pH 7.0, $[Fe^{3+}] = 10^{-18}$ M). Hence, significant concentrations of water-soluble Fe^{3+} species can be formed only by strong complex formation (Crichton 2001).
On the basis of the interconversion of iron oxidation states, this metal takes part in electron transfer and binds reversibly ligands as well. Its unoccupied d orbitals make it possible to bind ligands, preferring oxygen, nitrogen and sulfur atoms as biological ligands. Since Fe^{3+} is a hard acid it gives preference to hard oxygen ligands, whereas Fe^{2+} is on the edge between hard and soft, preferring nitrogen and sulfur ligands. All

interactions between Fe^{2+} and Fe^{3+} and ligand donor atoms are depending on the strength of the chemical bond formed between them (Crichton 2001).
The bounded ligand can modify the electronic spin state and biological redox potential, ranging from very positive to negative values (Nunez, Urrutia et al. 2012). All these potential features enable iron to participate in a large number of biochemical processes. The general classification of these processes is oxygen transport and storage, electron transfer and substrate oxidation-reduction.
Four main classes of iron-containing proteins execute these processes in the mammalian system: 1) iron-containing nonenzymatic proteins (hemoglobin and myoglobin), 2) iron-sulfur enzymes, 3) heme-containing enzymes and 4) iron-containing enzymes that are noniron-sulfur, nonheme enzymes. In the first class iron functions as a critical ligand for the binding of dioxygen, by playing a major role as part of hemoglobin that transports oxygen from the lung to tissues. In the second class iron participates in single-electron-transfer reactions primarily in energy metabolism. Also in the third class iron, bounded to various forms of heme, takes part in electron transfer reactions when associated with a variety of cofactors (e.g., cytochrome P450 complexes). The last category includes all processes in which iron is not bound to a porphyrin ring structure or in iron-sulfur complexes (for a detailed review, see (Webb 1992, Crichton 2001) .

1.1.2. The role of iron in the brain

Iron is essential in neurocognitive and neurobehavioral development. Data from animal studies have shown that iron deficiency in utero or in early postnatal life can result in abnormal brain structures, because this metal is necessary for correct neurogenesis and differentiation of certain brain cells and brain regions (Rao, Tkac et al. 2007, Ward, Tkac et al. 2007, Rao, Tkac et al. 2003). Two structures in the rodent brain, the hippocampus and the striatum, have been identified in these studies to show an altered morphology.
Studies that investigate brain iron function can be divided into those that focus on (a) monoamine metabolism, (b) GABA metabolism, and (c) oligodendrocyte metabolism and myelination (Beard, Connor et al. 1993). Data on these topics derive mainly from animal or cell culture studies.

1.1.2.1. Iron and neurotransmitters

The development of the dopaminergic system takes place rapidly during early postnatal life with a fast increase in the number and density of dopamine transporters, and receptors in terminal field up to early puberty. Furthermore, during this time period other monoamine transporters and receptors are also being actively expressed in developing neuronal tracts with constant modification in density up through puberty and into adulthood. These monoamine projections are essential in the organization of axonal growth and synapse formation during early stages of brain growth but switch to their more traditional role of neurotransmission with aging (Beard 2003).

The role of intraneuronal iron in metabolism is diverse and has been investigated by a number of research groups over the last four decades (Beard 2001, Beard 2003). As a result, it is generally known that iron is essential for a number of enzymes which are involved in neurotransmitter synthesis (Beard, Connor et al. 1993, Wigglesworth, Baum 1988) including tryptophan hydroxylase (serotonin), tyrosine hydroxylase (norepinephrine and dopamine) and xanthine oxidase (Youdim, Green 1978). Furthermore, iron is a cofactor for ribonucleotide reductase, and is important for the functioning of several electron transfer reactions associated with lipid metabolism as well as with brain-energy metabolism (Wigglesworth, Baum 1988). Iron is linked to the activity of monoamine oxidase which is critical in the catabolism of neurotransmitters in the dopaminergic, serotoninergic and noradrenergic systems of the brain (Youdim, Grahame-Smith et al. 1975, Youdim, Green et al. 1980). Variations in brain iron status have influence on levels of monoamines (Adhami, Husain et al. 1996, Ashkenazi, Ben-Shachar et al. 1982, Kwik-Uribe, Gietzen et al. 2000a, Nelson, Erikson et al. 1997). Nevertheless detailed mechanisms of effect are still being explored (Erikson, Jones et al. 2000, Wiesinger, Jones et al. 2002).

As mentioned before, iron deficiency is critical for neuroembryogenesis and physiology, furthermore regional brain iron deficiency seem to affect dopaminergic tracts (Ben-Shachar, Finberg et al. 1985, Erikson, Jones et al. 2000, Erikson, Jones et al. 2001, Erikson, Pinero et al. 1997, Erikson, Shihabi et al. 2002, Morse, Beard et al. 1999, Morse, Beard et al. 1999, Nelson, Erikson et al. 1997, Youdim 1990). Significantly lower densities of dopamineD2 receptors in the striatum of rats were demonstrated before, moreover recent studies demonstrate that nucleus accumbens

is also sensitive to the effects of iron deficiency (Erikson, Jones et al. 2001, Yehuda, Youdim et al. 1987, Yehuda 1990, Yehuda, Youdim 1989, Youdim 1990). Beside that, the dopamine transporter density is notably reduced in striatum and nucleus accumbens, both terminal fields of neurons originating in the substantia nigra and ventral tegmentum (Erikson, Jones et al. 2000). In vivo microdialysis studies show that extracellular dopamine is elevated in striatum of iron-deficient rats and returns to normal levels when brain iron content and iron status are again normalized (Chen, Jones et al. 1995, Nelson, Erikson et al. 1997). Pharmacological experiments with cocaine (a dopamine transporter inhibitor) indicate a reduced sensitivity, suggesting alterations in transporter density and functioning (Erikson, Jones et al. 2000). These findings may explain poor attention in iron-deficient infants (Angulo-Kinzler, Peirano et al. 2002, Lozoff, Jimenez et al. 2000, Roncagliolo, Garrido et al. 1998).
Appropriate rates of dopamine clearance from the interstitial space greatly affect the capability to process environmental information (Beard, Connor 2003). Many of the cognitive and behavioral tasks depend on adequate functioning of the nigrostriatal dopaminergic and mesolimbic pathways as well as the noradrenergic projected fields in the midbrain (Beard 2008). Dopamine, along with norepinephrine are seen as important potential biological explanations for human dysfunctions in motor control, sleep cycles and activity, as well as learning and memory (Lozoff, Beard et al. 2006). Consequently changes in dopamine metabolism in the mesolimbic and the nigrostriatal tracts could easily be linked to altered perception and motivation. Though, lesions in several other parts of the brain may also result in changes in perception, memory, and motivation. For that reason the specificity of the connection between striatal dopamine changes and impaired spatial memory, attentional deficits, and avoidance behavior needs further exploration (Beard, Connor 2003).
The serotonergic and noradrenergic systems are biochemically related to dopamine, and therefore sharing a family of monoamine transporters responsible for uptake of these neurotransmitters into presynaptic neurons (Beard, Connor 2003). It has not yet been completely clarified if these other monoaminergic systems are truly sensitive to changes in brain iron status (Adhami, Husain et al. 1996, Chen, Jones et al. 1995, Nelson, Erikson et al. 1997). Animal models have shown that the serotonin transporter density (Morse, Beard et al. 1999) and norepinephrine uptake rates (Beard, Chen et al. 1994) are altered by iron deficiency, whereas data on humans is

limited (Beard 2008). A previous study (Voorhess, Stuart et al. 1975) showed that urine of iron-deficient infants was particularly high in norepinephrine and returned to normal with the restoration of iron adequacy. A further study (Beard, Borel et al. 1990) showed changes in plasma norepinephrine levels in iron-deficient women during cold stress.
As mentioned before, it is important to keep in mind that serotonin, norepinephrine and dopamine transporters are all members of the same family of Na^+ cotransporters and show similar characteristics with regard to regulation and translocation (Reith, Xu et al. 1997).

1.1.2.2. GABA

Brain areas that receive γ-aminobutyric acid (GABA)eric innervations showed intense iron staining suggesting a probable connection between iron and this inhibitory neurotransmitter (Hill 1985). Youdim and colleagues (Youdim, Green et al. 1980) observed no effect of iron deficiency on the GABA receptor population, amount of GABA or production rates, whereas other investigators report that iron deficiency in utero and postweaning is related to major decreases in glutamate decarboxylase, glutamate dehydrogenase, and GABA transaminase activities (Li 1998, Taneja, Mishra et al. 1986). Glutamate dehydrogenase and GABA transaminase are shunt enzymes responsible for the synthesis and degradation of GABA. It has been shown that concentrations of GABA are elevated in dietary iron deficiency in hippocampus, striatum, and globus pallidus (Erikson, Shihabi et al. 2002) and may be associated with changes in brain manganese metabolism.
Nonetheless, further investigations are definitely needed to clarify the general role of iron in monoamine metabolism and GABA metabolism.

1.1.2.3. Oligodendrocyte metabolism and myelination

The necessity of iron for proper myelination of the spinal cord and white matter of cerebellar folds is generally known (Larkin, Rao 1990, Kwik-Uribe, Gietzen et al. 2000a). The oligodendrocyte is the predominant cell type containing iron in the human brain as well as in the mouse, rat, monkey, and pig brain (Connor, Fine 1987,

Hill 1988). These cells are responsible for the production of myelin, as well as for the synthesis of fatty acids (Mackler, Person et al. 1979, McKay, Higuchi et al. 1983) and cholesterol for myelin (Beard, Connor 2003).

Iron-containing oligodendrocytes appear next to neuronal cell bodies, along blood vessels, and are particularly plentiful within white matter tracts. Generally, iron-positive cells in white matter exist from birth and are finally located in defined patches of cells in the adult. It has been noticed that these patches of iron-containing cells usually have a blood vessel in their center. Throughout the second post-natal week, the distribution of iron-positive oligodendrocytes colocalizes with the myelinogenic foci, therefore a functional association between iron accumulation and myelin production is assumed (Connor, Menzies 1996). This functional association is consistent with the fact that the highest period of iron uptake in the CNS coincides with the peak myelination (Taylor, Morgan 1990, Connor, Menzies 1996). A possible explanation for the iron acquisition by oligodendrocytes at the peak of myelination is related to their energy metabolism. During this time of oligodendrocyte development, glucose is primarily metabolized via pentose-phosphate shunt, which offers reducing equivalents (NADPH) for synthesis of myelin fatty acids and also demands iron as cofactor for its key enzymes (i.e., glucose-6-phosphate dehydrogenase). When peak myelination is finished, pentose-phosphate decreases to 25% of total cellular metabolism, further followed by a reduction in iron consumption, but no reduction in iron staining or apparently iron utilization occurs (Cammer 1984).

As mentioned before, iron seems to be essential for optimal oligodendrocyte function, since both animal as well as human studies have observed hypomyelination in chronic severe iron deficiency. Both auditory brain stem potentials and visual evoked potentials are seen as indirect markers of myelination. Human studies have documented a delay in these auditory and visual evoked potentials in iron-deficient children compared to normal controls (Roncagliolo, Garrido et al. 1998, Algarin, Peirano et al. 2003). In several animal studies using the rat model of iron deficiency, restriction of dietary iron during gestation and the early post-natal period showed a decrease in myelin proteins (proteolipid protein and myelin basic protein), lipids (galactolipids and phospholipids) and cholesterol in iron-deficient animals compared to controls (Yu, Steinkirchner et al. 1986, Ortiz, Pasquini et al. 2004). Beard and colleagues (Beard, Wiesinger et al. 2003) put rats on an iron-restricted diet after the

post-weaning period (post-natal days 21–63) and observed also a significant decrease in myelination indices (including myelin basic protein, phospholipids, CNPase [2',3'-cyclic nucleotide 3'-phosphodiesterase] and cytochrome oxidase activity) in the hindbrain and cerebrum. These results indicate that oligodendrocytes of the adult brain are metabolically active and require constant iron delivery. The concept of constant iron delivery for continued myelin production and maintenance in adult is also accurate in humans (Bartzokis 2002).

Larkin and Rao (Larkin, Rao 1990) suggested that the common thread among animal studies exploring myelin and iron deficiency shows that although globally decreased, the relative ratio of myelin components is in fact normal. All these data further imply that the consequence of iron restriction is associated with a general decline of myelin production from a metabolic compromise and not a loss of one particular myelin component that is solely dependent on or regulated by iron. All in all, iron deficiency consequences on myelin production indicate that iron requirements for myelin are associated to metabolic processes underlying general myelin production rather than limited to lipid or protein biosynthesis. So far, it is still not clarified whether iron deficiency leads to global brain hypomyelination by altering oligodendrocyte number or their differentiation state or both (Todorich, Pasquini et al. 2009).

Morath and Mayer-Proschel (Morath, Mayer-Proschel 2001) showed that iron can alter oligodendrocyte proliferation and differentiation, but they also state that the timing of iron supply is critical for its effects on myelination.

When exploring the effects of iron deficiency on oligodendrocytes and its molecular mechanisms leading to decreased myelin, the best documented biological consequences are its post-translational effects on the iron-requiring proteins involved in energy metabolism and myelin synthesis (Beard, Dawson et al. 1996). As previously mentioned, iron is needed as a cofactor for cytochromes a, b, and c, and the Fe-sulfur complexes of the oxidative chain and is consequently absolutely necessary for adenosine triphosphate (ATP) production (Glinka, Gassen et al. 1997). Oligodendrocytes are seen as the most metabolically active cells in the brain, supporting myelin membranes that can exceed 100x the weight of an average oligodendrocyte (Cammer 1984). Therefore, oligodendrocytes are generating a relatively high supply of ATP, which could be an explanation for their sensitivity to iron deficiency. Various enzymes that are involved in ATP production as well as in

cholesterol and fatty acid synthesis, which are precursors of myelin are iron dependent. Following enzymes are included: HMG-CoA reductase, succinic dehydrogenase, NADH dehydrogenase, dioxygenase and glucose-6-phosphate dehydrogenase. Interestingly, most of these enzymes are enriched in oligodendrocytes compared to other cells in CNS (Pinero, Connor 2000). Other enzymes that also have iron as cofactor are lipid saturase and desaturase enzymes, which are involved in increasing and decreasing number of double bonds in fatty acids of lipids (Lange, Que 1998). Iron deficiency affects the function and degradation of these proteins, causing impaired energy production and consequently leading to a decrease in myelin production (Todorich, Pasquini et al. 2009). Several studies indirectly strengthen the relationship between iron and myelin, e.g. Wagner-Recio and colleagues (Wagner-Recio, Toews et al. 1991) showed that in humans, tellurium leads to peripheral neuropathy by inhibiting squalene epoxidase, a key enzyme in synthesis of cholesterol that needs iron as cofactor. An explanation for this inhibition was determined to be tellurium mimicking iron at key site of the enzyme and thereby inhibiting its function (Harry, Goodrum et al. 1989, Wagner-Recio, Toews et al. 1991). Moreover, exposure to ethanol during the development has many pathological effects including a delay in myelination and further ethanol in utero was related to iron deficiency in the brain. (Harris, Wilce et al. 2000, Ozer, Sarioglu et al. 2000, Zoeller, Butnariu et al. 1994, Miller, Roskams et al. 1995). In summary, it can be said that the common thread in various developmental models is that decreased iron availability to oligodendrocytes results in adverse outcomes on their function, development and myelination (Todorich, Pasquini et al. 2009).

All in all we can summarize that iron is essential for normal neuronal development, behavior and cognitive functioning. It is a fundamental cofactor in a variety of biological processes including myelin synthesis and neurotransmitter production and therefore indispensable for normal brain function.

1.2. Iron homeostasis

1.2.1. Dietary iron absorption

The human body contains 3-5 g iron (Ponka 1997), the vast majority, about 60–70% is present in hemoglobin in circulating erythrocytes. Further 10% of iron is present in the forms of myoglobins, cytochromes, and iron-containing enzymes, resulting in 4–8 mg of iron. The remaining 20–30% of iron is stored as ferritins and hemosiderins in hepatocytes and reticuloendothelial macrophages (Conrad, Umbreit et al. 1999).
In view of the fact that iron is needed for a number of diverse cellular functions, a constant balance between iron uptake, transport, storage, and utilization is required to preserve iron homeostasis (Lieu, Heiskala et al. 2001).
Both heme and non-heme iron is taken up by the gastrointestinal tract, primarily in the proximal small intestine, specifically in the crypt cells of the duodenum and jejunum (Conrad, Parmley et al. 1987, Wood, Han 1998) **(Figure 1)**. In the intestinal lumen iron is available in two forms as ferrous and ferric iron salts. To ensure efficient absorption of insoluble ferric iron, a prior reduction, mediated by a mucosal ferrireductase (Ekmekcioglu, Feyertag et al. 1996, Riedel, Remus et al. 1995) or chelation by amino acids or sugars must take place (Conrad, Umbreit et al. 1999). Ferric iron becomes insoluble at pH values above 3, whereas most ferrous iron remains soluble even at pH 7 (Conrad, Umbreit et al. 1999), explaining the better absorption of ferrous iron salts (Conrad, Weintraub et al. 1966).

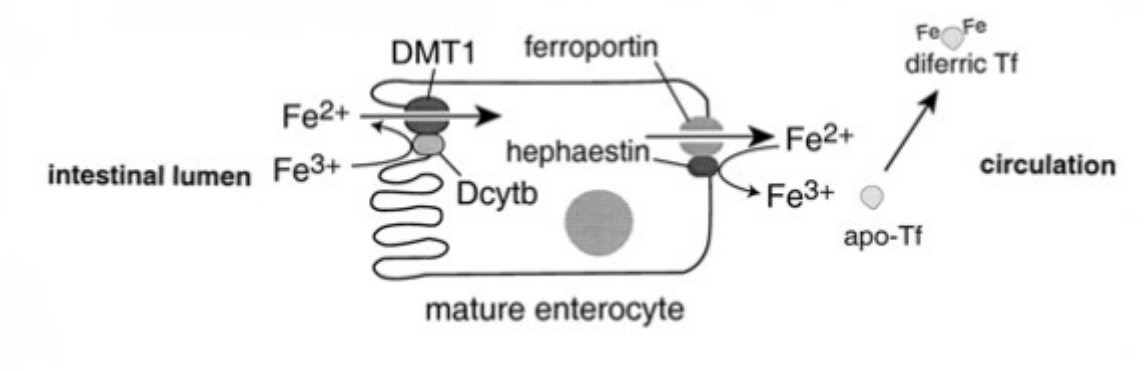

Figure 1. A model for iron transport across duodenal epithelial cells. Ferric iron is reduced to ferrous iron in the intestinal lumen by duodenal cytochrome B (Dcytb) and further transported across the apical membrane of the mature enterocyte by divalent metal transporter (DMT1). Iron export across the basolateral membrane of enterocytes to

circulation is mediated by ferroportin. This step is accompanied with reoxidation of ferrous to ferric iron by membrane bound hephaestin. In the circulation, plasma iron is directly scavenged by transferrin (Tf). Adapted from (Pantopoulos 2004).

Enterocytes are located on the intestinal villus, further they are very specialized, polarized absorptive cells that manage the passage of dietary iron in the lumen of the gut and the transport of iron into the body's circulation (Wood, Han 1998). Iron must cross three cellular barriers to enter the body's circulation: iron absorption across the apical membrane, intracellular iron translocation across the cytosol, plus iron export across the basolateral membrane into the circulation (Lieu, Heiskala et al. 2001). In contrast to other nucleated cells in the body, enterocytes contain no transferrin receptors (Pietrangelo, Rocchi et al. 1992); hence iron uses a mechanism that is different from the classical transferrin–transferrin receptor pathway for entering the cells (Lieu, Heiskala et al. 2001). A major protein that is most likely to be responsible for iron transport from the duodenum lumen into the cytoplasm of epithelial cells is a divalent metal transporter named DMT1/DCT1 (divalent cation transporter)/Nramp2 (natural resistance-associated macrophage protein) (Mims, Prchal 2005, Lieu, Heiskala et al. 2001, Fleming, Trenor et al. 1997, Gunshin, Mackenzie et al. 1997). An alternative pathway of iron uptake into enterocytes might be mediated by the paraferritin complex, consisting of *β* integrin, mobilferrin, and flavin mono-oxygenase (Conrad, Umbreit et al. 1999, Umbreit, Conrad et al. 1998).
After iron enters the enterocytes, the metal is subsequently exported into the circulation by an iron exporter know as ferroportin (Anderson, Vulpe 2009), which is located on the basolateral membrane of duodenal enterocytes (McKie, Marciani et al. 2000). It is believed that this iron exporter plays a key role in Fe^{2+} transport across the basolateral membrane of enterocytes in the gut by a mechanism that collaborates with the membrane-resident ferroxidase hephaestin and serum ceruloplasmin (McKie, Marciani et al. 2000). The protein hephaestin is very similar to ceruloplasmin, which is a multi-copper oxidase with ferroxidase activity that is necessary for the release of iron into blood and the binding to transferrin (Harris, Klomp et al. 1998). Hephaestin and ceruloplasmin are not transporters, but they assist the transport of iron from enterocytes into the body's circulation (Harris, Klomp et al. 1998, McKie, Marciani et al. 2000). Animal studies demonstrate that ceruloplasmin knockout mice have significantly impaired cellular iron export in reticuloendothelial cells (Harris,

Durley et al. 1999), as well as lower levels of hippocampal iron and reduced neurotransmitter levels (Texel, Camandola et al. 2012). The mechanisms between ferroportin and iron efflux across the basolateral membrane as well as the interactions with hephaestin and ceruloplasmin are yet not clarified (Lieu, Heiskala et al. 2001).
Further, latest research has also confirmed the importance of lysosomes, by showing their necessity in the release of iron from ferritin (Kidane, Sauble et al. 2006), as well as their contribution in the degradation of many iron-containing proteins. Consequently lysosomes are likely to contain high levels of mostly ferrous iron, because of the acidic environment (Terman, Kurz 2013). Moreover, this intralysomal iron is able to damage its membranes through lipid peroxidation, leading to the release of iron into the cytosol, resulting in a source for toxic iron (Hagemeier, Geurts et al. 2012).

1.2.2. Transferrin and transferrin receptors

Within the body, after absorption, iron is transported throughout the circulation bound to the glycoprotein transferrin (Tf) (Hagemeier, Geurts et al. 2012). Unlike enterocytes, the majority of non-intestinal cells acquire iron from transferrin (Lieu, Heiskala et al. 2001).
Generally, transferrin exists as a mixture of iron-free (apotransferrin), one iron (monoferric transferrin), and two irons (diferric transferrin or holo-transferrin) forms of the molecule. The concentration of iron and transferrin in blood plasma determines the relative amount of each form. Under normal conditions, the majority of the iron molecules in blood plasma are bound to transferrin, and iron–transferrin complexes enter cells using a transferrin receptor (TfR)-mediated endocytic pathway, explained below.

1.2.2.1. Transferrin receptors

The existence of two transferrin receptors (TfRs) known as TfR1 and TfR2, is well established (Kawabata, Yang et al. 1999). It is known that the well-defined function of transferrin receptor 1(TfR1) is to mediate cellular iron uptake from plasma transferrin (Lieu, Heiskala et al. 2001). TfR2 is a membrane protein equal to TfR1 (Herbison,

Thorstensen et al. 2009). Both receptors are expressed in the liver, while in contrast to the ubiquitous expression pattern of TfR1, TfR2 is mainly expressed in the liver, where expression of transferrin receptor 1 is comparatively low (Lieu, Heiskala et al. 2001, Kawabata, Yang et al. 1999). Further, there are many differences between TfR1 and TfR2. TfR1 has a higher affinity for diferric transferrin than TfR2 (West, Bennett et al. 2000, Kawabata, Germain et al. 2000) and TfR1 levels are regulated by cellular iron levels, whereas TFR2 levels are regulated by transferrin saturation (Robb, Wessling-Resnick 2004, Johnson, Enns 2004). Additionally, the expression of TFR2 is also regulated by the hemochromatosis protein (HFE) (Chen, Chloupkova et al. 2007).

In conclusion, it can be said that TfR1 and TfR2 are probably not only regulated through different pathways but also mediate iron uptake and storage by a different, yet not totally clarified, mechanism. It seems like that TfR1 plays a general role in cellular iron uptake, whereas TfR2 appears to play a specific role in iron uptake and storage in the liver, because of its high expression in hepatocytes (Lieu, Heiskala et al. 2001).

1.2.3. Transferrin-mediated cellular iron uptake

The main function of transferrin is to bind iron from plasma and to transport the metal to a variety of cells and tissues (Lieu, Heiskala et al. 2001).

Transferrin has the ability to carry two ferric iron (Fe^{3+}) atoms concomitantly, creating diferric transferrin (Fe_2–Tf) (Hagemeier, Geurts et al. 2012). To begin the process of cellular iron uptake, this protein complex binds to transferrin receptors located on the membrane of TfR-containing cells, initiating the transferrin-to-cell cycle (Zecca, Youdim et al. 2004a, Andrews 2000) **(Figure 2)**. In this procedure, endocytosis is started when diferric transferrin is invaginated into clathrin-coated pits (Hagemeier, Geurts et al. 2012), which fuse with the target membranes of endosomes supplying the vesicle contents into the inner of the endosome (Crichton, Dexter et al. 2011). An ATP-dependent proton pump reduces the pH of the endosome to around 5–6, which enables the release of iron as Fe^{3+} from the transferrin receptor complex. After reduction of Fe^{3+} to Fe^{2+} by a member of the Steap family of metalloreductases (Ohgami, Campagna et al. 2006), iron is transported by the divalent cation

transporter DCT1 or DMT1 out of the endosome into the cytoplasm, entering the intracellular labile pool, where it then can be transferred to the mitochondria for use in haem and iron–sulfur cluster synthesis, or be stored in ferritin. In contrast to most other protein ligands taken up by receptor-mediated endocytosis, apotransferrin is recycled back to the cell surface, for reuse, completing the cycle of cellular iron uptake (Crichton, Dexter et al. 2011, Lieu, Heiskala et al. 2001).

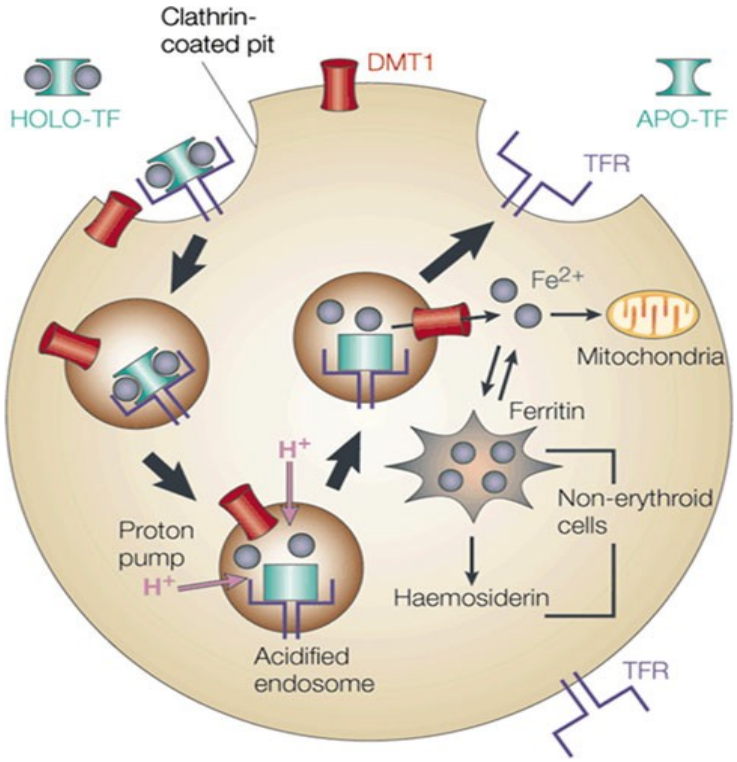

Figure 2. The transferrin cycle. Holotransferrin/diferric transferrin (HOLO-TF) binds to transferrin receptors (TFR) at the cell surface. The complex localize to clathrin-coated pits, endocytosis is started when the complex is invaginated. Thereby specialized endosomes are formed which will be acidified by a proton pump. When acidic pH is reached, iron is released from transferrin and is co-transported with protons out of the endosomes by the divalent metal ion transporter DCT1. Apotransferrin (APO-TF) bound to TFR is recycled back to the cell membrane, where, at neutral pH they dissociate to complete further rounds of iron delivery. In non-erythroid cells, iron can be stored as ferritin and haemosiderin (adapted from (Crichton, Ward 2006).

1.2.4. Ferritin

The most important iron-storage protein is ferritin, which is an essential detoxification machinery that prevents free iron from forming reactive oxygen species (Lieu, Heiskala et al. 2001). This molecule is composed of two subunits (H and L), heavy and light chains that form a protein shell that can store up to 4500 molecules of iron

(Theil 1998). The two subunits have different functions and they are also encoded by different chromosomes (Crichton 2001). The H-chains have a ferroxidase centre, which is able to catalyse the rapid oxidation of Fe^{2+} to Fe^{3+}. The L-chains are necessary for the nucleation of Fe^{3+} within the protein shell. It is known, that L-rich ferritins are associated with iron storage and stabilization of ferritin–iron units, whereas H-chain ferritins are associated with responses to stress (Koorts, Viljoen 2007). Storage of iron mainly occurs in the liver in macrophages and hepatocytes, typically in the form of ferritin.

1.2.5. Regulation of iron uptake and storage

The understanding of systemic iron homeostasis is nowadays increasingly described **(Figure 3)** and thought to be connected to a feedback mechanism where the peptide hormone hepcidin is secreted by hepatocytes. Increased levels of diferric transferrin, are detected by the liver via an as-yet unknown complex pathway involving HFE, TfR2 and hemojuvelin (HJV). Hepatocytes react to this signal by increased expression of the HAMP (hepcidin antimicrobial peptide) gene, leading to an increased secretion of hepcidin. High levels of hepcidin in the circulation, lead to a reduction of dietary iron uptake by blocking iron export through internalization of ferroportin in duodenal enterocytes and macrophages. When serum iron levels are appropriately reduced, hepcidin secretion will be also reduced, completing the feedback mechanism (Crichton, Dexter et al. 2011, Nemeth, Tuttle et al. 2004).

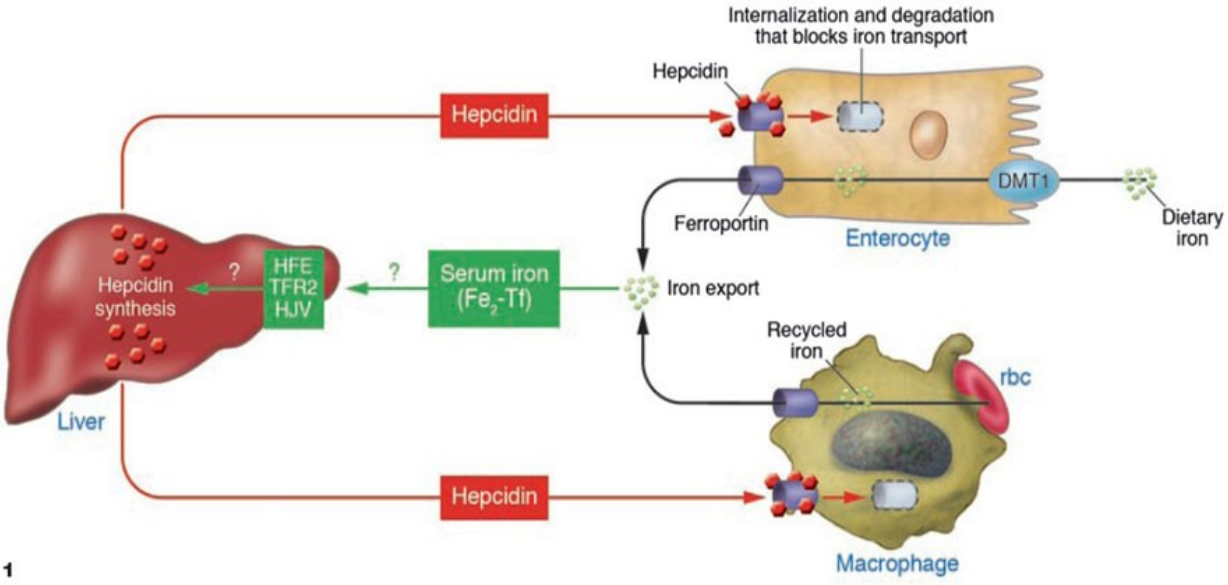

Figure 3. Regulation of systemic iron homeostasis. Increased diferric transferrin, Fe2–Tf, is detected by the liver through a regulatory pathway involving HFE, TFR2, and HJV. Hepatocytes act in response to this signal by inducing HAMP expression and hepcidin secretion. Circulating hepcidin acts in turn to reduce dietary iron absorption by the enterocytes and iron recycling by the macrophages via the internalization of ferroportin, which blocks iron export. This results in a decrease of serum iron. The feedback response includes the downregulation of hepcidin synthesis, which allows ferroportin molecules to be displayed on the surface of the target cells (Crichton 2008).

On the other hand, cellular iron homeostasis is to a great extent regulated at the level of the translation of the mRNAs of proteins involved in cellular iron metabolism. As soon as iron concentrations are diminished, the so-called iron regulatory proteins (IRP1 and IRP2), which function as cytosolic iron sensors, bind with high affinity to stem loops, known as iron regulatory elements (IREs) on the 5' untranslated region of ferritin and ferroportin mRNA and 3' untranslated region of transferrin receptor 1 mRNA (Crichton, Wilmet et al. 2002). This action leads to the translational suppression of ferritin and stabilization of TfR mRNA, resulting in the protection of TfR mRNA from nuclease degradation. These processes cause decreased iron storage in the form of ferritin and an increase in iron uptake by TfR. On the contrary, when iron is sufficiently present, iron regulatory proteins (IRPs) are detached from mRNA, leading to an increased translation of ferritin and ferroportin and enhanced nuclear degradation of TfR mRNA. These actions result in an increase in iron storage and efflux and a decrease in iron uptake (Crichton, Dexter et al. 2011) **(Figure 4)**.

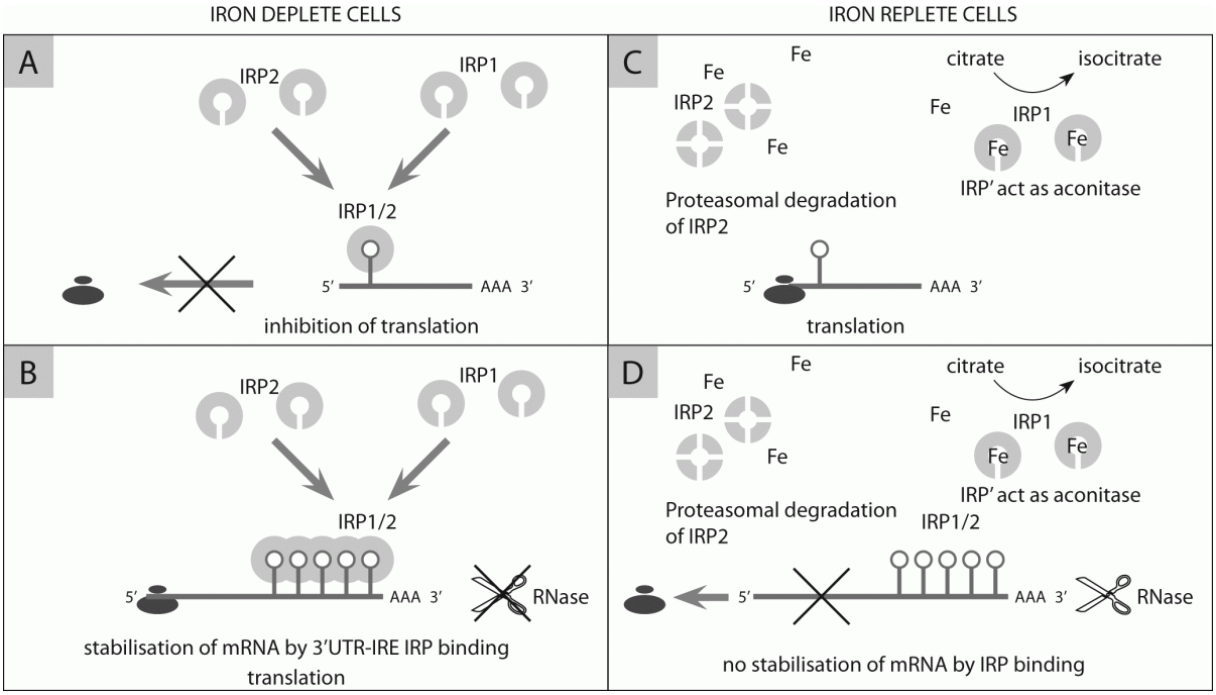

Figure 4. Maintenance of cellular iron homeostasis by IRE/IRP system. In cells with deplete iron binding of IRP1/2 at 5´UTR IRE of ferritin and ferroportin mRNA block translation inhibiting its initiation (A); The binding of IRP1/2 at 3´UTR IRE of TfR mRNA leads to stabilization of its transcript (B). In iron replete cells IRP1 act as aconitase and IRP2 is degraded by proteases so translation of 5´UTR IRE mRNA of ferritin and ferroportin is completed without disturbance (C). When iron is sufficiently present, there is no binding of IRP1/2 and stabilization of 3´UTR IRE transcript of TfR (D). Adapted from (Tandara, Salamunic 2012)

1.3. Brain iron metabolism

Mechanisms underlying the function of iron metabolism and homeostasis in the periphery have been elucidated enormously in the past years, while the understanding of iron metabolism in the brain is still under investigation (Altamura, Muckenthaler 2009).

1.3.1. The blood-brain barrier

The brain is a unique organ compared to all other organs of the body (Crichton, Dexter et al. 2011), separated from the systemic circulation by the blood-brain barrier (BBB), a tight structure consisting of endothelial cells, basal lamina, pericytes, and astrocytic foot processes that limits access to plasma nutrients, such as iron

(Altamura, Muckenthaler 2009). **Figure 5** displays the types of cells found at the BBB and their associations.

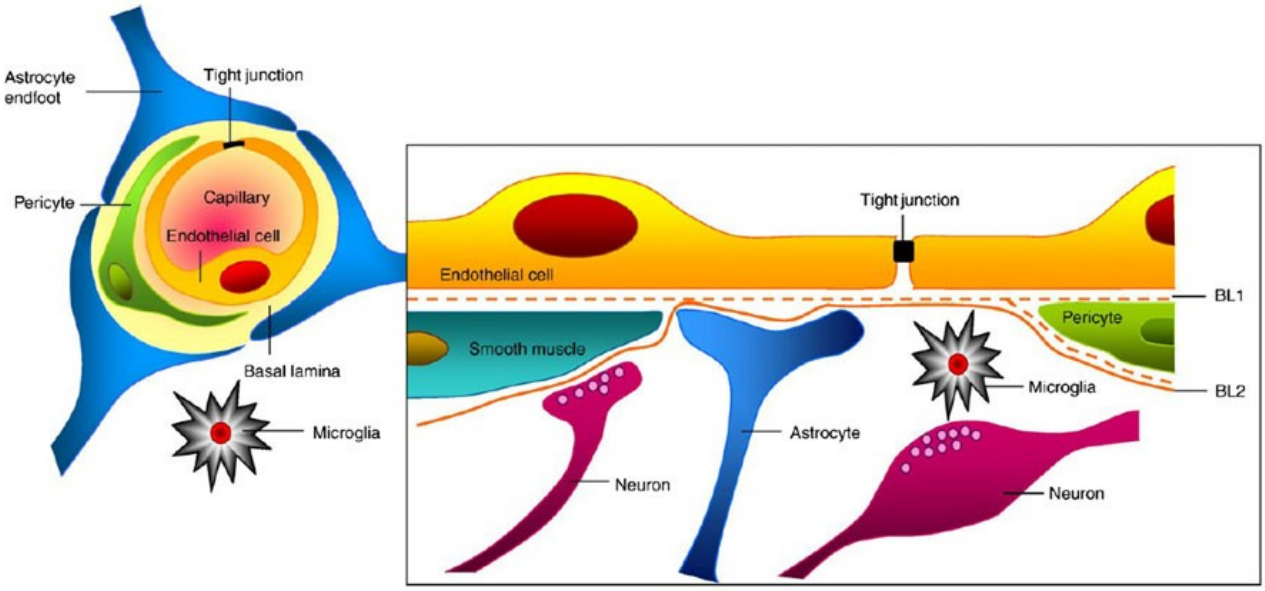

Figure 5. BBB cells and their associations (adapted from (Abbott, Patabendige et al. 2010).

It is supposed, that the main brain iron transport is managed through the transferrin-to-cell cycle, using transferrin receptors within epithelial cells lining the blood–brain barrier, even though the exact mechanism of iron transfer is still under discussion. Within the BBB, the endothelial cells form tight junctions which close the paracellular pathway between the cells, so that any substance which enters the brain must use dedicated endothelial cell transport systems. In contrary to other blood vessel epithelia, the BBB epithelia express other receptors at the luminal membrane, which is facing the circulation, compared to the abluminal membrane, surrounded by astrocyte end-feet, neuronal processes and interstitial fluid. Pericytes are spread along the length of the cerebral capillaries, to a certain extent surrounding the endothelium. There are two different basal laminae, basal lamina 1(BL1), a separate perivascular extracellular matrix formed by both the cerebral endothelial cells and the pericytes which are enclosed by the local basement membrane, and the extracellular matrix of the glial end-feet bounding the brain parenchyma (BL2). Further, foot processes from astrocytes form a complex network nearby the capillaries. In addition, microglia, which are the local immunocompetent cells of the brain, are also seen in

the surrounding area of the BBB. The blood-to-brain trafficking of solutes across the BBB may be enabled by passive or active transporters in the endothelial cell membranes. After crossing the BBB, diffusion distances for solutes to neurons and glial cells are not far, because no brain cell is farther away than about 25 µm from a capillary (Crichton, Dexter et al. 2011).

1.3.2. Iron transport into and within the brain

As mentioned above, it is still not completely clarified, how iron crosses the BBB. Since the brain, as the only organ in the body with this capacity, expresses transferrin receptors (TfR1) on the luminal side of its capillaries (Angelova-Gateva 1980, Jefferies, Brandon et al. 1984, Kawabata, Yang et al. 1999), it is believed that the major route of iron transport across the luminal membrane of the capillary endothelium is managed through the transferrin receptor-mediated endocytosis by brain capillary endothelial cells (BCECs) (Bradbury 1997, Moos, Morgan 1998b, Moos, Morgan 2000).
Quite a few other mechanisms, explaining how iron subsequently exits the BCECs and reach the brain extracellular fluid, are suggested, including a) receptor-mediated transcytosis of iron-containing transferrin (Descamps, Dehouck et al. 1996), b) release of iron from the endosome via DMT1 (Siddappa, Rao et al. 2002, Siddappa, Rao et al. 2003); as well as c) a role for low molecular weight molecules released from astrocytes that capture iron once it exits the BCECs (Moos, Morgan 2004, Moos, Skjoerringe et al. 2006).

1.3.2.1. Iron transport from blood to endothelium

The proposal of receptor-mediated transcytosis of iron-containing transferrin has been dismissed, since several studies showed no evidence for the transport of transferrin through BCECs (Crowe, Morgan 1992, Strahan, Crowe et al. 1992, Moos, Skjoerringe et al. 2006).
The existence of several observations indicates that transferrin receptors are indispensable for iron uptake by the brain (Moos, Rosengren Nielsen et al. 2007). During embryonic development, transferrin receptors are expressed by proliferating

neural progenitor cells (Copp, Estibeiro et al. 1992) and BCECs (Moos, Oates et al. 1998). Failure of transferrin receptor expression in fetal mice leads to lethal outcomes during development with severe defects in the CNS most likely caused by the shortage of iron uptake by dividing brain cells (Levy, Jin et al. 1999). While the transferrin receptor is constantly expressed by BCECs and neurons in the developing rodent brain (Moos, Oates et al. 1998), the expression pattern seems to be age-dependent with highest expression in BCECs around the second postnatal week. Taylor and Morgan (Taylor, Morgan 1990) showed that in neurons expression reaches its peak levels from the beginning of the fourth postnatal week, when the BBB integrity is completely developed and the rate of iron transport into the brain is lower than that of the developing brain. Further, in conditions with iron deficiency, the transport of iron into the brain is noticeably higher than in conditions with normal iron levels (Taylor, Crowe et al. 1991).

Against expectations, iron deficiency is not accompanied by higher expression of transferrin receptor protein in BCECs (Moos, Oates et al. 1998), probably explained by the suggestion that they raise the cycling rate of endosomes containing transferrin receptors instead (Moos, Rosengren Nielsen et al. 2007).

The initial step in the transport of iron from the luminal to the abluminal side of the BCECs includes the bond of diferric transferrin to transferrin receptors expressed at the luminal membrane of the BCECs, leading to a subsequent receptor-mediated endocytosis, and formation of endosome and finally resulting in the release of iron from transferrin within the endosome by the slightly acidic pH. Consequently, iron could then be released into the brain, with the simultaneously recycling of apotransferrin to the luminal side of the endothelial cells and its final release into the plasma (Moos, Morgan 2002, Rouault, Cooperman 2006). Although the role of DMT1 for the release of iron from the endosome is documented (Burdo, Menzies et al. 2001, Siddappa, Rao et al. 2002, Siddappa, Rao et al. 2003), there is still some controversy as unexpectedly DMT1 could not be detected in BCECs in a series of independent investigations, in spite of a ready detection in neurons and choroid plexus epithelial cells (Moos, Morgan 2004, Moos, Skjoerringe et al. 2006).

In this context the BCECs´ own obligatory need for iron should not be ignored, especially during brain development when BCECs are rapidly proliferating (Mato, Ookawara et al. 1989). Therefore, Moos and colleagues (Moos, Rosengren Nielsen

et al. 2007) proposed that the theoretical occurrence of DMT1 in BCECs, which may be too weak and too low to be detectable by immunoassays, relates to iron-transport into the cytosol to feed the BCECs themselves rather than playing a role in iron transport across the BCECs.

In conclusion, it has been proposed that BCECs mediate iron transport into the brain by separating iron from transferrin without the involvement of DMT1 (Moos, Skjoerringe et al. 2006) and that iron is transported from the luminal to the abluminal surface of these cells inside vesicles without any step which leads to its release from the endosome into the cytosol and from there into the brain interstitium (Moos, Rosengren Nielsen et al. 2007). Supporting evidence for this hypothesis would be the presence of transferrin receptor-containing vesicles at the abluminal side of BCECs, which has been indicated by several *in vivo* studies (Roberts, Fine et al. 1993, Bickel, Kang et al. 1994, Gosk, Vermehren et al. 2004), nevertheless convincing data are missing (Moos, Rosengren Nielsen et al. 2007), although *in vitro* studies do indicate that transferrin-containing vesicles fuse with the abluminal surface of BCECs (van Gelder, Cleton-Soeteman et al. 1997, Burdo, Menzies et al. 2001). If the fusion process of transferrin receptor-containing vesicles and the abluminal surface of BCECs indeed occur, iron, detached from transferrin and probably in its ferric form, would be available to be further transported into the brain, not requiring ferroportin, which is responsible for ferrying ferrous iron out of other types of cells such as neurons and oligodendrocytes (Wessling-Resnick 2006, Burdo, Menzies et al. 2001, Moos, Rosengren Nielsen 2006, Wu, Leenders et al. 2004), even though the oxidation state of iron transported by ferroportin is not yet definitely recognized (Moos, Rosengren Nielsen et al. 2007).

Wu and colleagues (Wu, Leenders et al. 2004) have discovered that BCECs express ferroportin, suggesting its involvement in exporting iron from the cytosol of the BCEC into the brain interstitial fluid (IF). To enable the latter binding of iron to transferrin, the multicopper ferroxidase ceruloplasmin (CP) that is attached to the end-foot processes of astrocytes via glycophosphoinositide (GPI) linkage, would prior convert ferrous to ferric iron. GPI–linked ceruloplasmin colocalizes on the astrocyte cell surface with ferroportin and is physically associated with it. Consequently, ferroportin alone is not capable to efflux iron from astrocytes in the absence of GPI–CP or CP (Jeong, David 2003). The ferroxidase activity is needed for the stability of cell surface

ferroportin in cells expressing GPI–ceruloplasmin (De Domenico, Ward et al. 2007). However, there is still disagreement about whether ferroportin is expressed in BCECs or not (Moos, Rosengren Nielsen 2006).

The interactions between BCECs and astrocytes probably represent another possibility for enabling iron transport into the brain (Moos, Rosengren Nielsen et al. 2007). After the receptor-mediated endocytosis of iron–transferrin at the luminal surface of the BCEC, the endosome, containing the transferrin-receptor complex, would be transported towards the abluminal side of the BCEC. There, still bound to its receptor, it would be subsequently exposed to a local microenvironment, leading to release of the iron. In the brain extracellular fluid a number of factors which can release iron from transferrin are present and may be at comparatively high concentrations near the BCEC–astrocyte endfoot junction (Crichton, Dexter et al. 2011). These iron releasing factors comprise hydrogen ions, ATP and other nucleotides, and citrate (Morgan 1977, Morgan 1979), which are released from astrocytes and other brain cells (Neary, Rathbone et al. 1996, Guthrie, Knappenberger et al. 1999, Montana, Malarkey et al. 2006).

After the release of iron from transferrin, apotransferrin would be again recycled to the luminal cell surface, leaving the acidic environment, where it would be released from the transferrin receptor and returned back to the circulation (Moos, Rosengren Nielsen et al. 2007).

Released iron would then pass into the brain interstitium to be bound by citrate, ascorbate (Bradbury 1997) or transferrin, which is present in the interstitial fluid (Moos, Rosengren Nielsen et al. 2007).

Figure 6 displays a diagrammatic portrayal of the BBB and the iron transport proteins thought to be involved in iron movement into the brain.

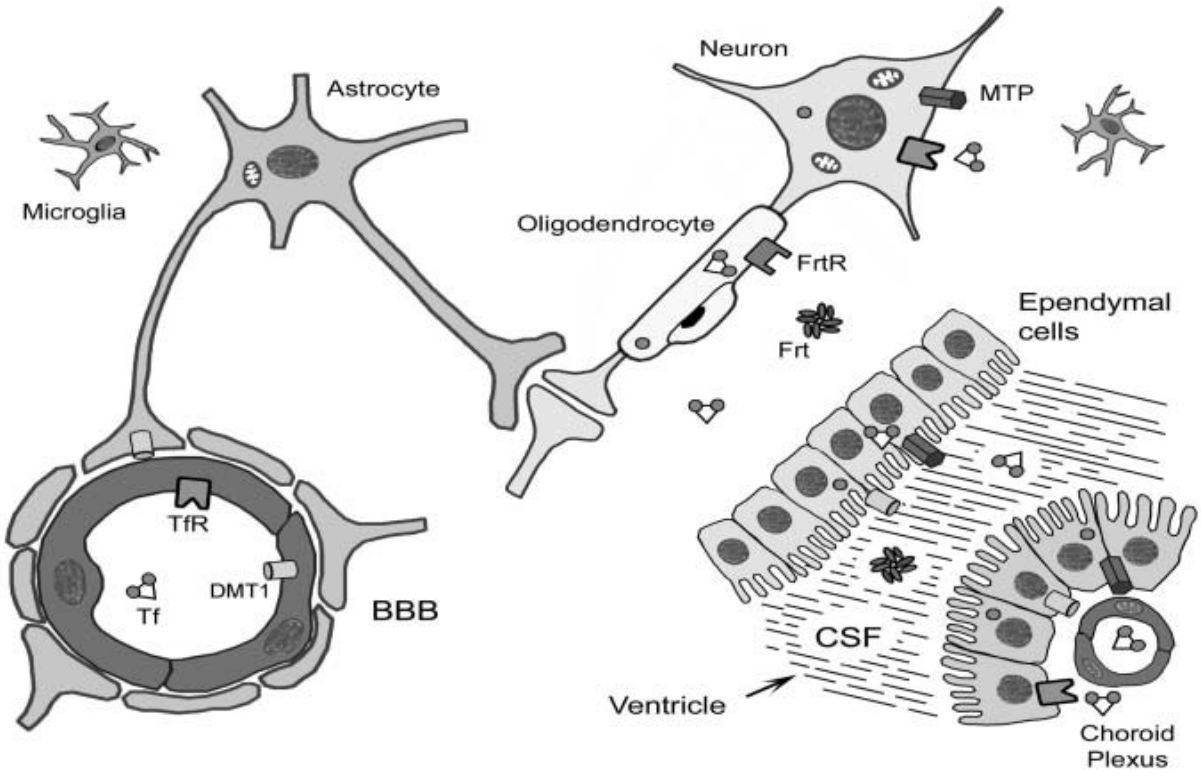

Figure 6. Diagrammatic portrayal of the blood-brain barrier and the iron transport proteins thought to be involved in iron movement into the brain. Abbreviations: DMT1 (divalent metal transporter 1), Tf (transferrin), TfR (transferrin receptor), MTP (metal transport protein or ferroportin), Frt (ferritin), FrtR (ferritin receptor), CSF (cerebrospinal fluid). Adapted from (Beard 2003).

1.3.2.2. The ventricular system

Most of the cerebrospinal fluid (CSF) is secreted by the choroid plexus into the ventricular cavities, while further possible sites of origin include the brain parenchyma and ependyma (McComb 1983). The circulation of the CSF in the ventricles and subarachnoid space, offers a great sink that serves to drain the interstitial fluid of the brain into the CSF (Moos, Rosengren Nielsen et al. 2007). Even though the CSF and the IF that bath the neurons are separated by different compartments, it is possible that the composition of CSF reflects that of the interstitial fluid (Bradbury 1997). Iron concentration in the CSF ranges between 0.2 and 1.1 µM while transferrin concentration is around 0.24 µM (Symons, Gutteridge 1998, Moos, Morgan 1998b). Like the endothelial cells of the BBB, also choroid plexus epithelial cells simultaneously form a barrier: the blood cerebrospinal fluid barrier (the blood–CSF

barrier) for circulating plasma proteins from the brain (Giometto, Bozza et al. 1990, Moos 1996). A main difference between the blood–CSF barrier and the BBB is that the choroid plexus of the lateral and third ventricle synthesize Tf, which may be important for transporting iron across the choroid plexus (Ke, Qian 2007). An additional difference is that iron is found in the choroid plexus epithelial cells of both developing and adult brain (Moos 2002). Therefore, it is hypothesized that Tf secretion from the choroid plexus into the CSF may also play a part in iron supply to brain parenchyma (Malecki, Cook et al. 1999). The iron content in choroid plexus epithelial cells goes along with a profound expression of ferritin mRNA and protein, which may indicate the existence of iron in the choroid plexus as non-transferrin-bound iron (NTBI), which is incorporated in ferritin, moreover it could result from detachment of iron from Tf following TfR-mediated endocytosis by choroid plexus epithelial cells (Moos 2002).
Additionally, the high rate of blood flow in choroid plexus has led to the theory that the majority of iron enters the brain through the CSF (Ke, Qian 2007), however observations of several studies (Ueda, Raja et al. 1993, Crowe, Morgan 1992) indicate that the contribution of iron transported through the blood–CSF barrier to the brain may be negligible as comparing the iron transport through the BBB (Ke, Qian 2007).

1.3.2.3. Circulation of iron in the brain interstitium

After the transportation of iron across the BBB or blood–CSF barrier, it is likely that iron binds quickly to Tf (Ke, Qian 2007). Within the brain the source for transferrin derives from diffusion from the ventricles (Szentistvanyi, Patlak et al. 1984, Bradbury 1997), as little transferrin is probably released from the abluminal surface of BCECs (Moos, Rosengren Nielsen et al. 2007), or transferrin is secreted by the oligodendrocytes and choroids plexus epithelial cells to supply neuronal cells and macro- and microglia with iron (Espinosa de los Monteros, Kumar et al. 1990, Bradbury 1997, Moos, Morgan 1998b).
The observations from several experiments display that the iron concentration exceeds that of the binding capacity of Tf in the CSF and interstitial fluid. Since the affinity of Tf with iron is the highest, in comparison to other iron transporters, ferric

iron in CSF and IF will bind to Tf first. In contrast to Tf found in the periphery, Tf in CSF and IF is fully saturated with iron, concluding that excess iron will bind to other transporters. Consequently, it is likely that there are two transport forms of iron in CSF and IF in the brain: Tf-Fe and NTBI (Bradbury 1997, Moos, Morgan 1998b). NTBI would be captured by provided molecules in the interstitial fluid, complexed to smaller organic molecules like citrate, ATP and ascorbic acid (Bradbury 1997).
A further potential extracellular iron carrier in the brain is lactoferrin, which is structurally closely related to transferrin but has an even higher affinity for ferric iron (Morgan 1981, Brock 1995). Lactoferrin is known to be synthesized in the mammary glands and neutrophil polymorphonuclear leukocytes and to circulate in low concentrations in blood plasma. Its role in the plasma transport of iron is not believed to be important, but this protein is involved in inflammatory reactions (Moos, Rosengren Nielsen et al. 2007). Further, it is present in the cerebrospinal fluid, in higher amounts after cerebral bleeding or infarction (Terent, Hallgren et al. 1981). On brain microvessels a lactoferrin receptors is present, and in vitro studies showed that lactoferrin can cross BCECs (Fillebeen, Descamps et al. 1999). The source of brain lactoferrin may derive from the blood, even though local synthesis is also possible (Siebert, Huang 1997).
Summarizing the above, it can be said that within the brain interstitium, iron would be transported bound to low-molecular weight constituents such as ATP and citrate (Bradbury 1997) as well as to large molecules like transferrin or lactoferrin (Fillebeen, Descamps et al. 1999). Transferrin and non-transferrin-bound iron are then taken up by neurons, astrocytes, and oligodendrocytes (Ke, Qian 2007).

1.3.2.4. Iron uptake by neuronal cells

1.3.2.4.1. Neurons

The widespread distribution of transferrin receptors and DMT1 in neurons clearly implies that neurons can take up iron-transferrin and transport it to endosomes from where it is pumped into the cytoplasm (Burdo, Menzies et al. 2001, Moos, Morgan 2004, Ke, Qian 2007). **Figure 7** displays a possible model for neuronal iron uptake and export. Under iron-deficient conditions, neurons potently up-regulate their

transferrin receptor expression, which emphasizes the importance of this receptor for iron uptake in neurons (Moos, Oates et al. 1998). Unlike the expression of DMT1 which remains at same levels in the brain during iron deficiency (Ke, Chang et al. 2005). Further, the occurrence of NTBI in brain CSF and IF indicates that neurons can acquire iron also by a transferrin independent pathway (Moos, Rosengren Nielsen et al. 2007), as they take up iron-citrate in culture conditions (Rosengren Nielsen and Moos, unpublished observation) (Moos, Rosengren Nielsen et al. 2007). The presence of lactoferrin receptors on neurons suggests that the lactoferrin/lactoferrin receptor -mediated pathway could represent a further possibility for neuronal iron-uptake (Ke, Qian 2007). The binding of lactoferrin to the lactoferrin receptor is independent of its degree of iron saturation (Davidson, Lonnerdal 1989).

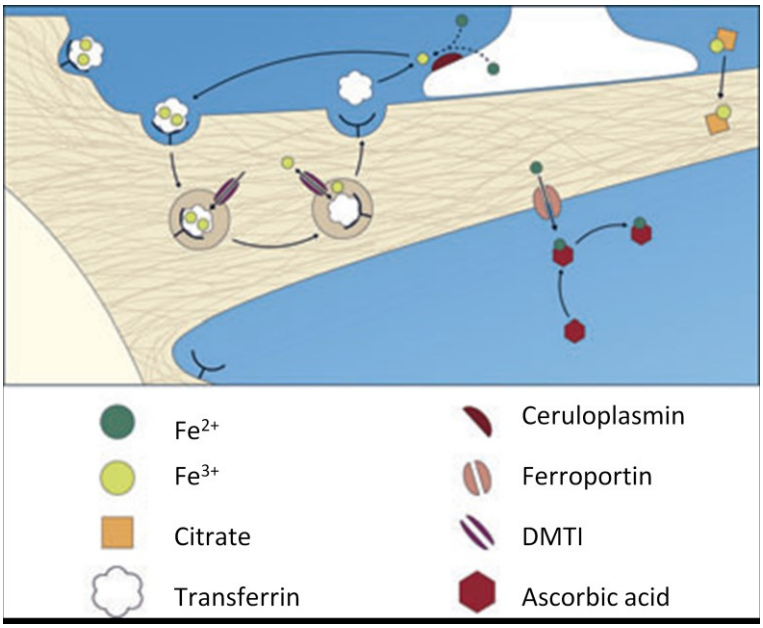

Figure 7. A model for neuronal uptake and export of iron. An astrocytic end-foot forming close contact with the neuron is also displayed. After the binding of iron-transferrin to the transferrin receptor at the cell surface, iron is transported into the neuron bound to transferrin. The resulting endosome contains divalent metal transporter 1 that assists iron transport across the endosomal membrane into the cytosol, at the same time pumping protons into the endosome. Astrocytes contain ceruloplasmin that demonstrates ferroxidase activity, which is able to oxidize ferrous iron to ferric iron. The emerging ferric iron can enter the neuron in a low-molecular weight form like iron bound to citrate or ATP. Neurons express the iron exporter ferroportin that transports ferrous iron out of the cell. In the interstitium ascorbic acid is present which can bind and thereby neutralize the toxicity of ferrous iron (adapted from (Moos, Rosengren Nielsen et al. 2007).

Generally, it is believed that neurons regulate their iron levels, and subsequently iron, not used for metabolic purposes, is released from the cells (Moos, Rosengren Nielsen et al. 2007). It has been observed that ferroportin is almost ubiquitously expressed in the brain suggesting that iron-export mediated by ferroportin is a permanently active mechanism, and thereby guaranteeing iron-homeostasis within the neuron (Wu, Leenders et al. 2004). Iron is assumed to go through axonal and dendritic transport, and as ferroportin is found in the somata, axons, and dendrites of neurons, it most likely has a significant function for regulating iron levels everywhere in the neuron (Moos, Rosengren Nielsen 2006). Interestingly, neurons of some forebrain nuclei also contain ferritin, indicating the capability of neurons for storing iron (Hansen, Nielsen et al. 1999).

As mentioned before, the presence of molecules with ferric reductase activity either in the plasma membrane or in the endosome/lysosome system is necessary to reduce ferric iron and transport it into the cytosol (Nunez, Urrutia et al. 2012). There are four membrane ferrireductases described which are involved in iron transport processes: Duodenal cytochrome B (Dcytb), its homologous cytochrome b561, Stromal cell-derived receptor 2 (SDR2) and 6-transmembrane epithelial antigen of the prostate 3 (Steap3) (Vargas, Herpers et al. 2003, Ohgami, Campagna et al. 2006). Dcytb was first described as the ferrioxidase, and it is responsible for the reduction of non-heme iron in the duodenal lumen during intestinal iron absorption (McKie, Barrow et al. 2001). A recent report showed the presence of Dcytb and SDR2 in astrocytes, where they seem to have a restricted role in iron accumulation (Tulpule, Robinson et al. 2010). There is no evidence for the presence of plasma membrane Dcytb in neurons. The high concentration of ascorbate in the CSF, thereby keeping NTBI in the ferrous state, could be a likely explanation for the absence of a membrane-bound iron reduction system in brain cells (Nunez, Urrutia et al. 2012).

In situ hybridization studies display that Steap2, a member of the Steap family of ferrireductases, is expressed in the brain (Ohgami, Campagna et al. 2006). A further study (Vargas, Herpers et al. 2003) found that choroid plexus and ependymal cells contain SDR2. Clearly more research is required to evaluate whether neurons contain a ferric reductase or not (Moos, Rosengren Nielsen et al. 2007). So far, the

role of ferrireductases in iron uptake by neurons remains speculative (Nunez, Urrutia et al. 2012).
However, it should be kept in mind that the expression of ferroportin may account for the export of residual iron from the neurons in the form of ferrous iron. There is still controversy whether the function of neuronal ferroportin depends on the cooperative function of iron oxidases like ceruloplasmin or hephaestin (Moos, Rosengren Nielsen et al. 2007). It is generally known, that ceruloplasmin is specifically expressed by astrocytes (Klomp, Farhangrazi et al. 1996), and further observations in ceruloplasmin null mice provided evidence of neuronal pathology (Jeong, David 2006).

1.3.2.4.2. Astrocytes

Under normal conditions (Connor, Benkovic 1992a) astrocytes do not express transferrin receptors, suggesting an iron-uptake mechanism that does not involve the classical transferrin–transferrin receptor pathway (Moos, Morgan 2004). Further, it has been demonstrated that astrocytes take up non-transferrin bound iron in vitro (Schipper, Bernier et al. 1999). Moos and Morgan (Moos, Morgan 2004) reported that DMT1 most likely is absent from astrocytes in vivo, whereas other investigators reported the presence of this transmembrane iron transporter in astrocytes (Huang, Ong et al. 2004, Huang, Ong et al. 2006). It is believed that ceruloplasmin which exhibits ferroxidase activity, is present on astrocytes in a membrane-bound form and is involved in the export of iron from these cells (Patel, David 1997). As mentioned above, Jeong and David (Jeong, David 2006) showed that the depletion of the ceruloplasmin gene in mice seem to affect astrocytes by accumulating iron and showing signs of pathological changes. These observations confirm the current hypothesis that ceruloplasmin is required for iron export from astrocytes (Klomp, Farhangrazi et al. 1996). It has been observed that the mutation of the ceruloplasmin gene leads to the unusual induction of ferritin expression by the affected astrocytes, an occurrence that is usually restricted to other cells including neurons, oligodendrocytes, and microglia. Further, the lack of ceruloplasmin also affected some neurons of the cerebellum, moreover it was suggested that the failure of astrocytes to oxidize ferrous iron could affect the iron availability for neurons, causing

them to become iron-depleted either by reduced iron concentration in the brain extracellular space or by impairment of a direct iron-transfer between astrocytes and neurons (Jeong, David 2006). It has been hypothesized that astrocytes form an important part of a so-called neurovascular unit formed between BCECs, astrocytes, and neurons (Abbott, Ronnback et al. 2006), and that they theoretically would take up iron directly from BCECs and direct it to neurons through intracellular transport. As discussed prior, iron deficiency causes an increase of neuronal transferrin receptors (Moos, Oates et al. 1998), however this was not found in ceruloplasmin null mice, therefore it is yet not justifiable (Moos, Rosengren Nielsen et al. 2007) to conclude that neurons suffered from a loss of direct iron-supply from damaged astrocytes caused by the lack of ferroxidase activity, as this was recently suggested by Jeong and David (Jeong, David 2006).

1.3.2.4.3. Oligodendrocytes

The importance of transferrin for survival, growth, and maturation of oligodendrocytes has been considered and demonstrated for almost three decades in both *in vivo* (Adamo, Paez et al. 2006, Badaracco, Ortiz et al. 2008, Escobar Cabrera, Zakin et al. 1997, Espinosa-Jeffrey, Kumar et al. 2002, Saleh, Espinosa de los Monteros et al. 2003) as well as *in vitro* (Espinosa de los Monteros, Foucaud 1987, Paez, Garcia et al. 2006) studies.
Nevertheless, comparable to astrocytes, oligodendrocytes are also likely to receive iron by a mechanism that does not involve transferrin receptors (Moos, Rosengren Nielsen et al. 2007) **(Figure 8)**. Furthermore, transferrin receptors are not discovered in corpus callosum and mature oligodendrocytes in the adult brain even in conditions of iron deficiency (Han, Day et al. 2003, Hulet, Powers et al. 1999), in addition, oligodendrocyte cultures deprived of transferrin continue to obtain iron (Takeda, Devenyi et al. 1998). These studies implicate a nontransferrin mediated iron uptake mechanism in the cells of oligodendrocyte lineage (Todorich, Zhang et al. 2011).

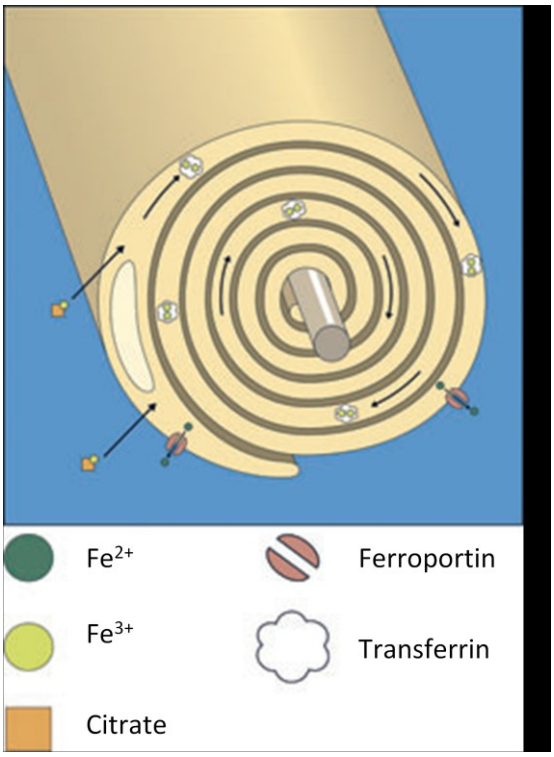

Figure 8. A schematic portrayal of possible iron uptake and transport in oligodendrocytes. Iron is taken up by the oligodendrocyte as low-molecular weight iron, here displayed as iron-citrate and becomes integrated in transferrin synthesized by the oligodendrocyte. The iron-transferrin is subsequently transported to distant areas of the oligodendrocyte. The oligodendrocyte also contains ferroportin that enables ferrous iron export (adapted from (Moos, Rosengren Nielsen et al. 2007).

A possible way could involve the uptake of iron-citrate, which subsequently leads to iron incorporation into endogenously expressed Tf (Bloch, Popovici et al. 1985) which can transport the metal to other compartments of the cell (Bloch, Popovici et al. 1985), whereas the secretion of transferrin by oligodendrocytes is also questioned (de Arriba Zerpa, Saleh et al. 2000). It could be possible that transferrin in oligodendrocytes serves as a molecule for intracellular iron transport along the extreme extensions, which wind around several axons (Moos, Rosengren Nielsen et al. 2007).

A recent study (Todorich, Zhang et al. 2008) has found ferritin receptors on oligodendrocytes and it has been suggested that H-ferritin (HF) displays a major iron source for oligodendrocytes and their subsequent ability to generate and maintain myelin (Todorich, Zhang et al. 2011). The authors conclude that since HF receptors are expressed on oligodendrocytes and because ferritin can transport as much as

100x the amount of iron per mole in comparison to transferrin (Harrison, Arosio 1996), this protein could represent the main biological iron delivery system for oligodendrocytes.
It is known that oligodendrocytes increase their iron as well as ferritin content with increasing age (Benkovic, Connor 1993), maybe thereby reflecting a deficiency in their ability to release iron, probably explained by the gradual loss of their capability to transfer iron back from the peripheral extensions to the soma (Moos, Rosengren Nielsen et al. 2007). Unlike astrocytes, oligodendrocytes have ferroportin, indicating that they are able to export iron as part of their regulation of intracellular iron homeostasis (Wu, Leenders et al. 2004, Moos, Rosengren Nielsen 2006). Further studies that observe the expression pattern of ferroportin in oligodendrocytes of the aging brain are needed to see if oligodendrocytes increase their ferroportin expression to compensate for increased iron levels or whether decreased expression may lead to iron accumulation in these cells (Moos, Rosengren Nielsen et al. 2007).

1.3.2.4.4. Microglia

Microglia derive from bone marrow cells of the myelomonocytic lineage, consequently during brain development the cells migrate into the brain from the circulation as monocytes, and then differentiate into resting microglia (Milligan, Cunningham et al. 1991). In this process the migrating cells are ferritin-containing because of a high iron content that is required for the production of free radicals as part of the so-called respiratory burst activity, but during their gradual transformation they lose their iron content and corresponding ferritin expression (Moos 1995, Cheepsunthorn, Palmer et al. 1998). When these cells reach their resting and fully differentiated state, they hardly ever contain histological detectable levels of iron, transferrin, ferritin or other iron-related proteins such as transferrin receptors or DMT1 (Moos, Rosengren Nielsen et al. 2007). In addition, unlike monocytes and macrophages, microglia do not contain ferroportin (Moos, unpublished observation) (Moos, Rosengren Nielsen et al. 2007). It has been demonstrated that microglia take up NTBI in culture (Takeda, Devenyi et al. 1998).

1.3.3. Iron efflux from brain cells

It is generally known that the quantity of brain iron increases with age but not as fast as would be expected from measurement of the rate of iron uptake from the blood at different ages (Morgan 1999). Therefore it can be concluded that some iron must be exported out of the brain to remain the brain iron homeostasis. It has been suggested that iron returns back to the circulation via the absorption of cerebrospinal fluid from the subarachnoid space (Moos, Morgan 1998b). Moos and Morgan (Moos, Morgan 1998a) demonstrated this in rats by the injection of radiolabeled transferrin into the lateral cerebral ventricle. The study displayed that transferrin was mainly quickly reabsorbed into the blood stream but a small proportion slowly entered the brain parenchyma and was capable to donate iron to brain cells. Consequently, it has been suggested that transferrin in the brain interstitial fluid could move in the reverse direction carrying with it iron derived from brain cells especially from cells which have the iron exporter ferroportin, such as neurons and oligodendrocytes, and export this metal to the ventricles or subarachnoid space (Moos, Rosengren Nielsen et al. 2007). Transferrin, present in the CSF, is completely saturated with iron (Bradbury 1997, Moos, Morgan 1998b). However, in the CSF, the concentration of transferrin is very low so that the capacity to export iron by this pathway is restricted (Moos, Rosengren Nielsen et al. 2007). Other routes for iron efflux from brain cells may include lactoferrin, ferritin and non-protein-bound iron which are also present in the CSF (Terent, Hallgren et al. 1981, Moos, Morgan 1998b). On the other hand, under normal circumstances the quantities of iron involved are very small but possibly can increase significantly under pathological ones (Moos, Rosengren Nielsen et al. 2007).

A further possibility for iron export involves the endothelial cells of the BBB which also have a potential capacity to mediate the export of elemental iron from brain interstitium to the systemic circulation, even though there is no evidence yet for direct iron export from the brain (Rouault 2001).

Also microglia and other phagocytic cells, which are able to enter the brain in inflammatory conditions and then exit the brain, are further important mediators of iron export after cell death and intracerebral hemorrhage that increases the local iron concentration significantly (Hua, Nakamura et al. 2006).

As discussed before, ceruloplasmin is involved in iron release from cells (Richardson 1999, Floris, Medda et al. 2000, Klomp, Farhangrazi et al. 1996), moreover clinical data on aceruloplasminemia demonstrate the potential role of ceruloplasmin in the release of iron from brain cells (Miyajima, Fujimoto et al. 1998, Miyajima, Takahashi et al. 1997, Gitlin 1998). Nonetheless, the exact mechanism of this ferroxidase and whether this particular process requires an iron transporter is not clarified. The iron transporter ferroportin has been identified in neurons and oligodendrocytes, but the necessity for the cooperative action of ceruloplasmin or hephaestin is under discussion. All in all, iron efflux from brain cells remains unresolved (Moos, Rosengren Nielsen et al. 2007).

1.4. Brain iron accumulation and the aging brain

1.4.1. Brain iron accumulation as a function of age

The progressive accumulation of iron in the brain as a function of age is generally accepted (Zecca, Youdim et al. 2004a). The first publication about iron accumulation in the human brain goes back to 1886 when Zaleski presented iron in a single human brain using the Perl´s stain (Koeppen 1995). His findings including the conclusions that the stains he observed were nonheme iron, and further it was bound to organic substances, were confirmed by others. Other researcher such as Guizetti and Spatz discovered that the staining was most clear in deep grey matter (GM) structures of the extrapyramidal system (Koeppen 1995). An important publication in 1958 by Hallgren and Sourander displayed that nonheme iron, mainly in the form of ferritin, was clearly higher in older people, in the putamen, caudate, globus pallidus, substantia nigra, dentate and thalamus, as well as the prefrontal, sensory, cerebellar and motor cortices. On the contrary, iron contents of the putamen, caudate nucleus and motor cortex increase to some extent slower, with highest levels being reached in old age (Hallgren, Sourander 1958). Additional latest research has confirmed that brain iron levels, as well as ferritin and transferrin, are altered during aging (Hagemeier, Geurts et al. 2012). Oligodendrocytes are the most prominent cells to stain for these molecules (Connor, Menzies et al. 1990). Connor and colleagues (Connor, Snyder et al. 1995) found that there is a relatively high level of heavy chain

(H) ferritin compared with light chain (L) ferritin in younger individuals; nevertheless both H- and L-ferritins increase with age in the frontal cortex, caudate, putamen, substantia nigra and globus pallidus.

Within the human brain the most highly pigmented cells are the dopaminergic neurons of the substantia nigra (SN) and the noradrenergic neurons of the locus coeruleus (LC). These cells contain the pigment which is composed of neuromelanin, a polymer formed by oxidized metabolites of dopamine, containing a peptide component of about 15% (Zecca, Tampellini et al. 2002). The iron-storage protein ferritin is poorly expressed in melanized dopaminergic neurons of the SN, when comparing with neurons in other parts of the brain (Snyder, Connor 2009). Hence, neuromelanin in the SN and LC is the location of main iron storage, also demonstrating increased levels during aging (Zecca, Gallorini et al. 2001). Neuromelanin eagerly binds iron in its ferric form, showing high and low affinity binding sites (Double, Gerlach et al. 2003). It is supposed that the high affinity sites are protective as they sequester iron in a redox-inactive form, while iron in the low affinity sites remains redox-active (Gerlach, Riederer et al. 2008). Under physiological conditions iron should securely bind to high affinity sites whereas in the case of increased age or pathophysiological conditions when iron concentration increases above the high affinity binding capacity of neuromelanin, iron binds to low affinity binding sites in a redox-active form resulting in the vulnerability of neurons by oxidative damage (Nunez, Urrutia et al. 2012). Zecca and colleagues (Zecca, Stroppolo et al. 2004b) observed several findings that indicate a difference in iron homeostasis between the LC and the SN in the brains of older individuals. Iron homeostasis is attained more efficiently in the LC than in the SN, observing a linear increase in iron concentration with age in the SN, while iron concentration in the LC is lower and remains steady throughout life. In the SN, H- and L-ferritin concentrations display an increase with age, while in the LC both ferritin subunits remain lower and invariant (Zecca, Stroppolo et al. 2004b, Zecca, Gallorini et al. 2001). Further findings include the observation that the neuromelanin concentration was similar in both regions, but the iron content in neuromelanin of the LC was much lower than in the SN. These results indicate that the iron mobilization and toxicity are lower in LC neurons than in SN neurons, leading to the conclusion that the bigger

damage occurring in SN could be related to the higher iron content (Zecca, Stroppolo et al. 2004b).

Additional investigations (Hirose, Ikematsu et al. 2003) discovered an increase in heme oxygenase-1 (HO-1), an essential enzyme involved in cellular metabolism, in the cerebral cortex and hippocampus as a feature of normal aging. This enzyme degrades heme and generates biliverdin (a powerful antioxidant), carbon monoxide and iron (Maines 1988). It is suggested that the increased expression of HO-1 in the aging human brain could lead to decreased resistance to oxidative stress, even though the enzyme promotes certain neuroprotective effects (Hirose, Ikematsu et al. 2003, Schipper, Song et al. 2009).

Brain iron content varies between cell types and brain regions, showing high concentrations in oligodendrocytes, particularly in the cortex and cortico-subcortical junction, and low concentrations in neurons and astrocytes (Connor, Menzies et al. 1990). Microglia demonstrate varying iron levels, because they store and release iron according to tissue metabolic needs (Connor, Menzies 1990). Notably, iron accumulation takes place within areas of myelination as well (Todorich, Pasquini et al. 2009).

Generally, brain iron accumulation starts at birth, when essentially no iron can be measured (Schenck, Zimmerman 2004, Taylor, Morgan 1990, Zecca, Gallorini et al. 2001) and increases rapidly during the first two decades of life in brain regions including globus pallidus, dentate, substantia nigra (pars reticulate), and red nucleus (Brass, Chen et al. 2006), but remaining relatively constant from 30 years onward and reaching maximum levels in old age (Bartzokis, Beckson et al. 1997). Interestingly, the thalamus accumulates iron to a minor extent (Brass, Chen et al. 2006), even showing a slight reduction in later life (Bartzokis, Beckson et al. 1997, Hallgren, Sourander 1958). Why iron accumulates in the brain with increasing age, is hardly known. There are some theories to explain this fact suggesting a dysfunction of the blood brain barrier (Farrall, Wardlaw 2009, Faucheux, Bonnet et al. 1999) or an accumulation caused by apoptotic cascade or cellular damage (Matsunaga, Kotamraju et al. 2004) such as cortical pathology, demyelination or white matter lesions (Dwork, Lawler et al. 1990). Others suggest age-related vascular changes and mitochondrial dysfunction leading to hypoxia, which may contributes to cellular

iron uptake through activation of hypoxia-inducible-factor-1 (HIFI) (Lee, Andersen 2006, Qi, Jamindar et al. 1995).

There exist general agreement that the mechanisms of how iron moves through the brain and accumulates within defined regions like the basal ganglia, and SN (Aoki, Okada et al. 1989, Bartzokis, Beckson et al. 1997) still need to be investigated. A number of observations indicate that the brain iron homeostasis includes efficient iron recycling mechanisms: a) brain iron uptake is most prominent during fetal life and iron repletion at the postnatal stage is unable to correct cognitive defects that develop in utero as a result of iron deficiency(Kwik-Uribe, Golub et al. 2000b); b) the rate of daily iron uptake into the brain is not as high than its requirements propose; and c) the brain iron content is nearly unchanged in adult mice that develop severe iron deficiency on a low-iron diet (Beard, Felt et al. 2006). Such a mechanism will protect the brain from systemic iron deficiency as well as iron overload in agreement with the notion that iron overload conditions like hereditary hemochromatosis do not have an effect on the brain iron content (Golub, Germann et al. 2005).

1.4.2. The aging brain

The previous section discussed the progressively accumulation of brain iron and the age-related changes in brain iron levels. Beside the gradually accumulation of brain iron with increasing age, further changes in the brain are considered as “normal” as we grow older.

In post-mortem and in vivo studies, comparison between brains of older and younger adults revealed that older adults tend to have lower volumes of grey matter (Haug, Eggers 1991, Resnick, Pham et al. 2003). These volume declines in older adults seem to arise from lower synaptic densities and not from cell death (Terry 2000). According to Terry and colleagues (Terry, Katzman 2001) neocortical synapse density declines progressively between the ages of 20 and 100, furthermore, by extension, synaptic density in non-demented older humans would reach the reduced density observed in Alzheimer’s disease by the age of 130. Nevertheless, regional changes in volume are not homogeneous, with some regions, such as the prefrontal cortex (PFC) and medial temporal structures, being mainly affected by either normal

or pathological ageing, and other regions, such as the occipital cortex, remaining comparatively unaffected (Resnick, Pham et al. 2003, Raz, Gunning-Dixon et al. 2004, Raz, Rodrigue et al. 2003, West 1996). It is suggested that there are two components with regard to the differential effects of ageing on specific brain regions (Gabrieli 1996). The first component contains alterations in the frontostriatal system, with reductions in dopamine, noradrenalin and serotonin, and declines in the volume and function of the PFC (Raz, Gunning-Dixon et al. 2004, Volkow, Wang et al. 1996). In addition, frontal white matter tracts experience an age-related loss of integrity that probably affects memory circuits involving the frontostriatal cortices (Head, Buckner et al. 2004). All these changes are seen in healthy individuals without dementia symptoms or hypertension as well, and develop steadily throughout adulthood and are associated with age-related declines in behavioral memory measures (Hedden, Gabrieli 2004).

The second component contains alterations that arise principally with pathology linked to Alzheimer's disease, beginning with a loss of volume in the entorhinal cortex, a significant relay between the hippocampus and association cortices, and gradually affecting the hippocampus proper. The development from healthy ageing to Alzheimer's dementia takes place in a subtle and graded fashion for maybe a decade or longer, as pathological changes in the entorhinal cortex start before clinical diagnosis of Alzheimer's disease (Dickerson, Goncharova et al. 2001, Killiany, Gomez-Isla et al. 2000).

Neuropsychologists speculated that prefrontal deficits were the primary cause of cognitive ageing (West 1996, Moscovitch, Winocur 1995), and that structures of the PFC experience the largest age-related volumetric alterations in adulthood (Haug, Eggers 1991, Resnick, Pham et al. 2003, Raz, Gunning-Dixon et al. 1998), with an estimated average linear decline of about 5% for each decade after the age of 20 (Raz, Gunning-Dixon et al. 2004). It has been observed that in healthy older adults, the largest declines in volume are found in lateral regions of the PFC (Tisserand, Pruessner et al. 2002), whereas patients with Alzheimer's disease demonstrate the greatest degeneration in the inferior PFC (Salat, Kaye et al. 2001), even though decline of the PFC is not detected early in the disease (Thompson, Hayashi et al. 2003). These volume declines are possibly explainable, to some extent, by reduced synaptic density in the PFC with ageing, which is documented in both monkeys

(Bourgeois, Goldman-Rakic et al. 1994) and humans (Liu, Erikson et al. 1996). Moreover, smaller, but consistent, age-related declines have been observed in the human striatum, a brain region that has extensive connections to the PFC and is needed for a large proportion of dopamine production, and could probably therefore influence cognitive processes that are subserved by dopamine-dependent circuits (Hedden, Gabrieli 2004).

Beside volumetric alterations, also a variety of neurotransmitters in the PFC and striatum experience age-related changes such as a reduction in dopamine concentration (Goldman-Rakic, Brown 1981), transporter availability (Volkow, Wang et al. 1998) and dopamine D2 receptor density (Volkow, Wang et al. 1996, Volkow, Wang et al. 1998). A further decline related to age is seen in serotonin receptor availability in the frontal cortex, which is, in addition, associated with striatal declines of dopamine receptors (Wang, Volkow et al. 1995), even though serotonergic declines appear to be largest in mid-life, with minor declines in later life (Sheline, Mintun et al. 2002).

Both volumetric and neurotransmitter alterations in the PFC and the striatum have been linked to age-related declines in cognitive performance; for further details, please see (Hedden, Gabrieli 2004).

The effect of ageing influences not only grey matter density, but also white matter density and the number of white matter lesions (Chen, Li et al. 2001, Guttmann, Jolesz et al. 1998). Several studies found the greatest age-related alterations in white matter in the PFC and the anterior corpus callosum (Head, Buckner et al. 2004, Bartzokis, Cummings et al. 2003), even though all brain regions experience some age-related decline in white matter integrity (Head, Buckner et al. 2004, O'Sullivan, Jones et al. 2001). Generally, white matter abnormalities are linked to poor performance on tasks of processing speed, executive function and immediate and delayed memory, but not with declines in general intelligence measures (Gunning-Dixon, Raz 2000). Further, the loss of white matter integrity with age has possibly an effect on the interaction of the PFC with structures such as the hippocampus and striatum (Hedden, Gabrieli 2004).

To further understand memory changes with advancing age, the investigation of brain regions such as the hippocampus and related medial temporal lobe (MTL) structures are of importance (Erickson, Barnes 2003). As opposed to the relatively

large age-related alterations arising in the PFC and frontal white matter tracts, studies of the human hippocampus and adjacent MTL structures have found comparatively slight age-related alterations in the absence of Alzheimer's disease (Raz, Gunning-Dixon et al. 2004). Summarizing the results of numerous studies on this topic revealed that normal ageing has minimal structural consequences on the hippocampus and adjacent MTL, even though age-related functional alterations possibly affect circuits that involve interactions between the PFC and the hippocampus, in that way leading to age-related declines in memory function that is mediated by hippocampal neurons. On the contrary, pathological processes begin by affecting the entorhinal cortex, in this manner disabling hippocampal participation in memory (Hedden, Gabrieli 2004).

Nonetheless, it should be mentioned that a common finding in cognitive ageing is that declines in function are likely to be accompanied by increases in variability (Ylikoski, Ylikoski et al. 1999). In general, there is variability across individuals within ageing populations and these individual differences are likely caused by variability in the severity of deficits in the two components mentioned before. Individual differences in normal ageing could possibly caused by variability in PFC integrity, and individuals could also vary in their susceptibility to the pathology of Alzheimer's disease (Hedden, Gabrieli 2004).

1.5. Iron Toxicity

The previous sections discussed the importance of iron for neuronal development and its indispensability for normal brain function. Although essential for life, excessive levels of iron can have devastating effects (Thompson, Shoham et al. 2001) such as the aggregation of α-synuclein in neurons which is associated with several neurodegenerative diseases, including Parkinson disease (Kostka, Hogen et al. 2008). Increasing amounts of low molecular weight, redox-active iron are potentially neurotoxic through the ability to induce oxidative reactions (Gutteridge 1992, Zecca, Youdim et al. 2004a, Richardson 2004, Honda, Casadesus et al. 2004) and by catalyzing the formation of reactive oxygen species (ROS) such as hydroxyl radicals (OH·) (Hagemeier, Geurts et al. 2012). The resulting oxidative stress can lead to the oxidation of cellular components causing the modification of DNA, proteins, lipids,

and carbohydrates, and the subsequent oxidative damage is often connected with cell death either by necrosis or by apoptosis (Dalle-Donne, Giustarini et al. 2003).

1.5.1. Oxidative stress and its detrimental consequences

In the Fenton reaction, ferrous iron (Fe^{2+}) is oxidized by hydrogen peroxide (H_2O_2) to ferric iron (Fe^{3+}), generating a hydroxyl anion (OH^-) and the reactive hydroxyl radical ($OH\cdot$).

$Fe^{2+} + H_2O_2 \rightarrow OH\cdot + OH^- + Fe^{3+}$ Fenton chemistry

Further, ferric iron (Fe^{3+}) can be reduced to ferrous iron (Fe^{2+}) by the superoxide ($O_2\cdot^-$) radical ($O_2\cdot^-$) ($Fe^{3+} + O_2\cdot^- \rightarrow Fe^{2+} + O_2$). The combination of these two reactions results in the so-called Haber-Weiss reaction ($O_2\cdot^- + H_2O_2 \rightarrow O_2 + OH\cdot + OH^-$), which leads to the generation of more hydroxyl radicals (Halliwell 1992, Jomova, Valko 2011). These hydroxyl radicals are the most reactive free-radical species known and have the ability to react with plenty of cellular constituents including amino-acid residues, and purine and pyrimidine bases of DNA (Crichton 2001) as well as promoting or exacerbating protein misfolding and aggregation (Andersen 2004). These radicals generate further free radicals by taking an electron from a polyunsaturated fatty acid (RH) in the brain:

$RH + OH\cdot \rightarrow R\cdot + H_2O$

This reaction transforms polyunsaturated fatty acids into organic free radicals ($R\cdot$) which may react with oxygen (O_2) to form an organic oxygen radical ($RO_2\cdot$). The oxygen radical may again cause damage by stealing an electron from another biological molecule (RH) to form an organic hydroperoxide (ROOH) and another radical to go on with the cycle.

$R\cdot + O_2 \rightarrow RO_2\cdot$

$RO_2\cdot + RH \rightarrow ROOH + R\cdot$

The organic oxygen radical is able to continue damage by reacting with other lipids through a chain reaction resulting in the oxidative destruction of lipids (Gutteridge 1992, Brass, Chen et al. 2006). This process is known as lipid peroxidation and leads

to a loss of membrane permeability to calcium with the associated cellular toxicity (Brass, Chen et al. 2006). Brain tissues such as cell membranes are rich in polyunsaturated fatty acids and therefore at risk of damage related to lipid peroxidation. In this process iron can be a destructive catalyst because the metal can be both initiate and increase lipid peroxidation (Gutteridge 1992). This oxidative chemistry is suggested to play a key role in deleterious free radical-mediated brain injury in both normal cerebral aging and neurological diseases (Brass, Chen et al. 2006).

Another source of free radicals derives from the non-enzymatic oxidation of dopamine in dopaminergic cells mediated by redox-active iron, leading to the production of semiquinones and H_2O_2 (Zoccarato, Toscano et al. 2005).

Also other iron compounds such as free hemoglobin is able to catalyze peroxidation of purified arachidonic acid and other polyunsaturated fatty acids within normal cell membranes in the presence of H_2O_2 and $O_2\cdot^-$ (Sadrzadeh, Graf et al. 1984). Additional research has demonstrated that the addition of purified hemoglobin, even in the absence of H_2O_2 and $O_2\cdot^-$, can result in brisk peroxidation of lipids in murine brain homogenate (Sadrzadeh, Eaton 1988). It has also been observed that the hemoglobin and/or iron-mediated reactions in the brain are catalyzed paradoxically by the otherwise antioxidant compound, ascorbic acid, and are blocked by an iron chelator such as desferrioxamine (Sadrzadeh, Anderson et al. 1987).

The reactive nitrogen species (RNS) nitric oxide NO, formed from the nitric oxide synthase (iNOS), has diverse functional roles such as acting as an antimicrobial agent in the cell. Its reaction with superoxide will form the peroxynitrite species ($ONOO^-$), which is very toxic to the cell. This non-radical product is protonated to peroxynitrous acid (ONOOH) at physiological pH. The chemistry of peroxynitrite/peroxynitrous acid is highly complex, even though addition of ONOO to cells and tissues results in oxidation and nitration of proteins, DNA and lipids with a reactivity that is similar to that of hydroxyl radicals (Crichton 2001).

Once ROS are generated by redox trace metals close to membrane phospholipids, they initiate the peroxidation of polyunsaturated acyl chains of phospholipids or n-6 polyunsaturated fatty acids (PUFA). The lipid hydroperoxides are highly vulnerable, resulting in the formation of various lipid-derived α, β-unsaturated 4-hydroxyaldehydes of which the most well-known is 4-hydroxynonenal (HNE). HNE is

the main aldehyde that is produced by lipid peroxidation in cells and it was initially accepted as the product of lipid peroxidation with the most toxicological potential. Consequently, HNE is seen as one of the most reliable markers of oxidative stress (Crichton, Dexter et al. 2011). Immunohistochemical studies show that HNE occurs in diverse neurodegenerative diseases such as Alzheimer's disease (AD), sporadic amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD) and in diffuse Lewy bodies disease (DLBD) (Zarkovic 2003). This hydroxyalkenal has been proposed to be one of the leading mediators of the damage resulting from exposure to reactive oxygen and nitrogen species (Crichton, Dexter et al. 2011).

In the presence of oxidative stress several post-translational modifications of proteins have been characterized resulting either from direct oxidation of amino acid residues by ROS that are formed throughout normal metabolism or through the conversion of lipid and carbohydrate derivatives to compounds that react with functional groups on proteins (Grimsrud, Xie et al. 2008). An important part of these ROS-induced post-translational modifications leads to the formation of reactive protein carbonyl derivatives, generally named ‘‘protein carbonylation’’. The level of carbonyl groups in proteins is commonly seen as a marker of oxidative protein damage (Berlett, Stadtman 1997, Beal 2002).

ROS can also willingly attack DNA, producing many DNA lesions, such as oxidized bases, abasic sites, and single and double-strand breaks. If these lesions are not correctly removed, they can be potentially dangerous, resulting in mutagenesis and/or cell death, particularly in the case of lesions that block the progression of DNA/RNA polymerases (Crichton, Dexter et al. 2011).

Oxygen-derived free radicals as well as the generation of RNS take place in the course of normal aerobic life. It is generally known that free radicals are involved in a large number of normal biochemical reactions; both reactive oxygen and nitrogen species are involved in cell regulation where oxidants and redox status are essential factors in signal transduction. Further, free-radical oxygen species are implicated in enzyme-catalyzed reactions, in mitochondria as by-products of the consumption of molecular oxygen in the electron transport chain, in signal transduction and gene expression, and in activation of nuclear transcription factors. The necessity of RNS as messengers in cells is also recognized. Throughout normal cellular metabolism, these radicals will quickly detoxify; nevertheless, if to some extent higher levels are

formed, these could initiate cellular toxicity as well as acting as signaling molecules to mobilize specific cellular defense mechanisms (Crichton 2001).

1.5.2. Cellular defense mechanisms

As mentioned above, ROS and RNS are generated within the cell as part of normal cellular mechanisms, therefore the cell is adequately provided with a variety of cytoprotective enzymes including catalase, superoxide dismutase and glutathione peroxidase, as well as antioxidants, such as glutathione (GSH) and α-tocopherol, which defend the cell against oxidative stress.

The tripeptide glutathione can exist in its reduced (GSH) or its oxidized disulfide form (GSSG) and it is the most abundant and major antioxidant agent in the central nervous system, where it reaches millimolar concentrations in the cytoplasm (Meister, Anderson 1983, Dringen, Gutterer et al. 2000). The ratio GSH/GSSG is an accurate reflection of the redox state of the cell (Schafer, Buettner 2001). Nunez and colleagues (Nunez, Gallardo et al. 2004) reported that iron accumulation leads to the consumption of GSH and the production of GSSG. Further data (Nunez, Urrutia et al. 2012) suggest the hypothesis that a decrease in GSH levels is a consequence of the increased oxidative load produced by increased ROS and by the consumption of GSH by iron during its redox cycling.

As previously mentioned, heme oxygenase-1 (HO-1) is an inducible enzyme that degrades heme to biliverdin, carbon monoxide and iron. Its activation is a ubiquitous cellular response to oxidative stress (Ryter, Tyrrell 2000). This enzyme is considered to be cytoprotective against oxidative stress and its expression has been seen in microglia, astrocytes and neurons in various pathological alterations in the brain (Crichton, Wilmet et al. 2002), although its activity could lead to potential pro-oxidant consequences (Ryter, Tyrrell 2000).

Further response mechanisms to higher levels of ROS include changes in gene expression in the cell such as transcriptional changes (Crichton 2001). NFKappaB (NFκB) was the first transcriptional factor in eukaryotic cells that responded directly to oxidative stress induced by hydrogen peroxide from which hydroxyl radicals were generated via Fenton chemistry (Schreck, Rieber et al. 1991). Most cell types have NFκB is in their cytosol as an inactive heterodimer bound to a third subunit IκB. This

transcriptional factor plays a main role in the regulation of various genes involved in pathogen responses and cellular defense mechanisms (Crichton, Wilmet et al. 2002). As mentioned before, increased accumulation of tissue iron has been related to pathogenesis in numerous diseases even though the degree of any toxicity will, to a certain extent, be dictated by the localization of the iron complex within the cell, i.e., cytosolic or lysosomal, its biochemical form, i.e., ferritin or haemosiderin (Ward, Legssyer et al. 2000), as well as the capability of the cell to prevent the generation and propagation of free radical species by the broad range of antioxidants and cytoprotective enzymes present in that cell (Ward, Abiaka et al. 1994) **(Figure 9)** . In particular, brain cells including neurons, astrocytes and microglia, demonstrate a decreased ability to respond adequately to oxidative stress, mainly with respect to their levels of glutathione and glutathione peroxidase, such that alterations in their iron status may predispose them to iron-induced oxidative stress (Crichton, Wilmet et al. 2002). Because brain iron stores increase significantly with ageing (Zecca, Gallorini et al. 2001), it can be logically concluded that increased iron, if localized in vulnerable regions, could contribute to the pathogenesis in a range of neurodegenerative diseases (Crichton, Wilmet et al. 2002).

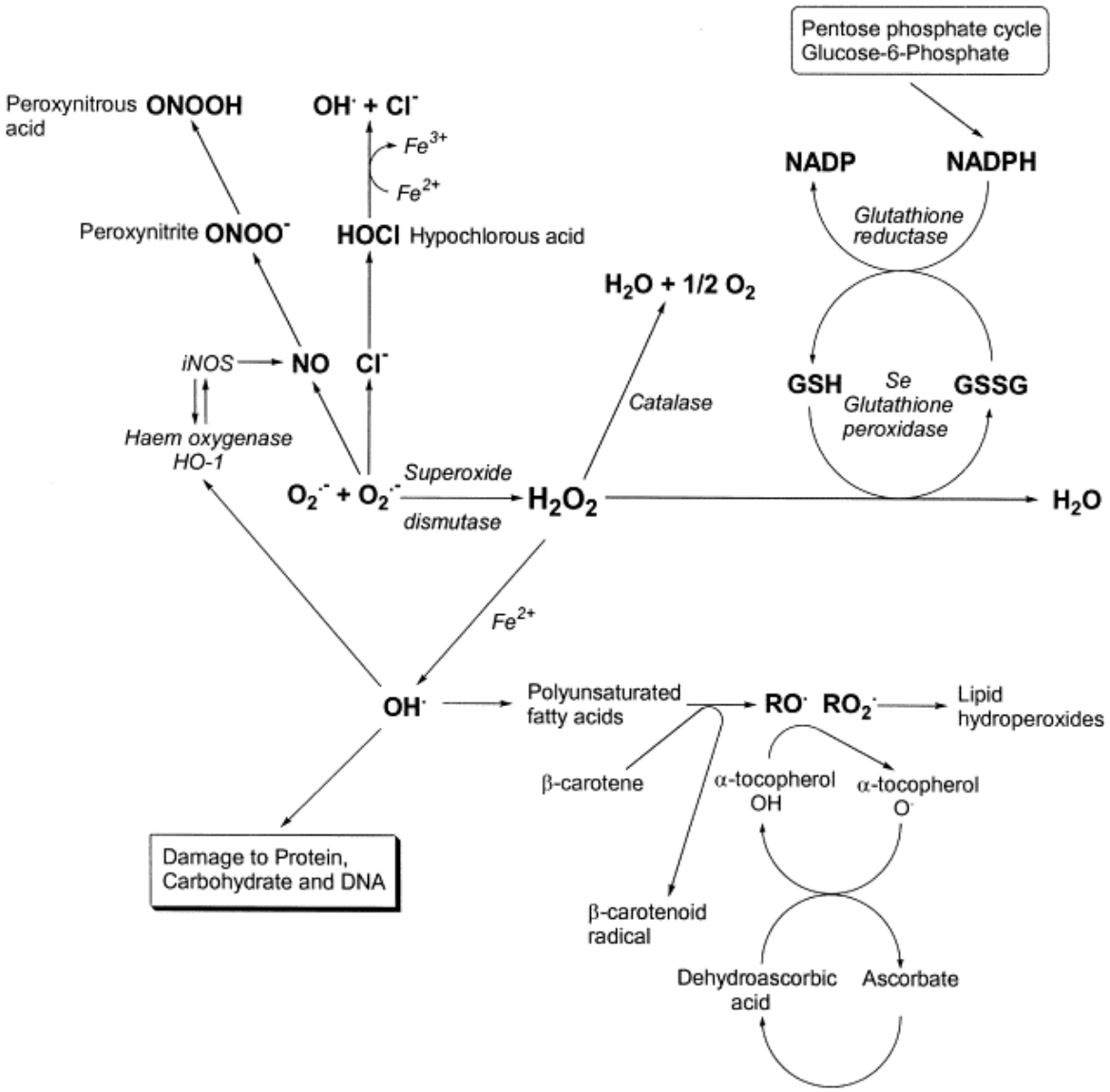

Figure 9. Protective mechanisms including cytoprotective enzymes and antioxidants act co-operatively in the form of a cascade scavenging reactive oxygen and reactive nitrogen species (adapted from (Crichton 2001).

1.6. Iron and neurodegenerative diseases

There is an increasing body of evidence that iron accumulation in specific brain regions is a common feature of a number of neurodegenerative and inflammatory disorders of the central nervous system such as Alzheimer's disease, Parkinson's disease, Huntington's disease (HD), and Friedreich's ataxia (FA) (Crichton, Dexter et al. 2011, Zecca, Youdim et al. 2004a, Brass, Chen et al. 2006). Generally, two classes of iron-related neurodegenerative disorders are distinguishable, those that arise from iron accumulation in specific brain regions, and those that arise from

defects in iron metabolism and/or homeostasis (Zecca, Youdim et al. 2004a). In this section all iron-related findings that are associated with the most common neurodegenerative diseases are discussed.

1.6.1. Non-genetic factors and disturbed iron regulation in the brain

Gene mutation or absence may not always be responsible for iron misregulation in the brain but probably non-genetic factors as well as exogenous factors such as aluminum (Yamanaka, Minato et al. 1999) should also be considered. It is suggested that these at present unknown non-genetic factors probably disturb normal control mechanisms of protein expression (Qian, Shen 2001, Qian, Wang 1998). The irregular distribution of brain iron and the different sites of increased iron accumulation in the various neurodegenerative disorders imply that there are a number of genes (or proteins) responsible for iron regulation in the brain (Roy, Andrews 2001, Qian, Shen 2001). Possible targets for the misregulation of iron in the brain include iron transport proteins such as lactoferrin receptor, and DMT1, as well as iron regulatory proteins, and other iron metabolism proteins (e.g. heme oxygenase-1) (Ke, Ming Qian 2003) **(Figure 10)**.

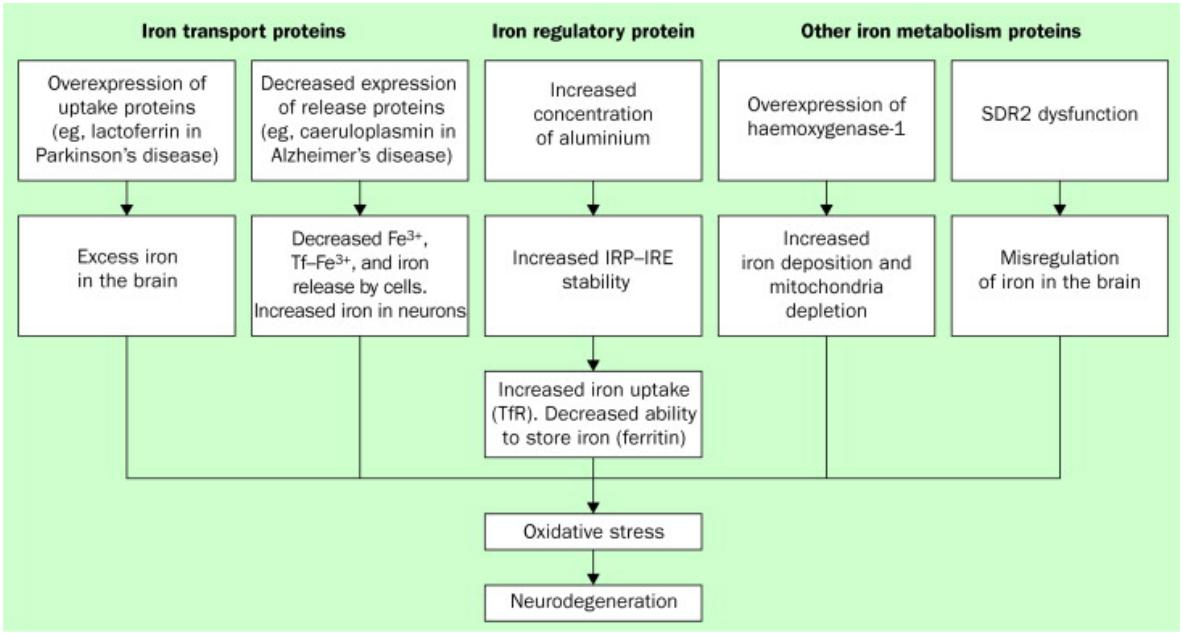

Figure 10. The probable involvement of non-genetic factors causing misexpression of iron metabolism proteins in the development of some neurodegenerative disorders. Adapted from (Ke, Ming Qian 2003).

Additionally, stromal-cell-derived receptor 2 (SDR2), a homologue of cytochrome b-561 and duodenal cytochrome b, was lately detected in human beings and mice; further a dysfunction of its catecholamine-regulated ferric reductase activity in the brain has been suggested to play a potential role in the progression of neurodegenerative disorders (Ponting 2001).

1.6.2. Alzheimer´s disease

With growing life expectancy in Western societies also the development of dementia which can progress to AD is increasing. Currently, over 24 million people worldwide suffer from some form of dementia, and it is estimated that, by 2040, 80 million people will be demented, with AD, accounting for some 60% of all dementias. AD is the most widespread cause of age-related neurodegeneration (Crichton, Dexter et al. 2011). It was first described in 1906 by the German physician Alois Alzheimer in a patient with strange behavioral symptoms, short-term memory loss, and a progressive decline of cognitive and motor function (Alzheimer 1907). The progressive loss of cognitive and behavioral function is related to the temporal and frontal lobes of the brain (Bush, Tanzi 2002). This disease is classically hallmarked by the presence of toxic insoluble aggregates of amyloid-β peptide (Aβ) in extracellular senile plaques and of neurofibrillary tangles (NFT) created by the hyperphosphorylation and subsequent aggregation of the microtubule-associated protein, tau, connected with the loss of cortical neurons (Honda, Casadesus et al. 2004). The initial step that drew attention to the link between iron and AD was the observation of iron accumulation in the same brain regions that are characterized by Aβ deposition, such as hippocampus, parietal cortex, and motor cortex (Good, Perl et al. 1992, Dedman, Treffry et al. 1992, Connor, Snyder et al. 1992c). Additionally, iron accumulation was also documented in neurons with NFT (Smith, Harris et al. 1997) and further, it has been observed that the binding of ferric iron and tau protein precedes the aggregation of hyperphosphorylated tau and the subsequent formation of NFT (Yamamoto, Shin et al. 2002). Also the existence of altered iron metabolism in AD, including alterations in iron, transferrin, and ferritin are well documented (Connor, Menzies et al. 1992b, Connor, Snyder et al. 1992c, Good, Perl et al. 1992, Thompson, Markesbery et al. 1988). It has been proposed that abnormal deposition

of iron in AD plaques related to β-amyloid can mediate free radical-related neurotoxicity (Smith, Harris et al. 1997).

Aβ is a 40-42 amino acid peptide processed by β- and γ-secretases from the amyloid- β protein precursor (AβPP), which is a type 1 transmembrane glycoprotein. The metalloprotein Aβ binds transitional metal ions through 3 histidine residues located in the N-terminal domain (Nakamura, Shishido et al. 2007, Atwood, Scarpa et al. 2000). Metal binding supports Aβ aggregation (Bush 2003, House, Collingwood et al. 2004), and in the absence of metal ions Aβ is not able to aggregate (Barrow, Yasuda et al. 1992, Barrow, Zagorski 1991). The protein AβPP is also cleaved by the α-secretase within the Aβ domain, destroying the Aβ sequence and producing and releasing the sAβPPα fragment with neuroprotective function **(Figure 11)**. The function of the α-secretase is modulated by furin, an enzyme belonging to the subtilisin- like proprotein convertase family which catalyses the cleavage of precursor proteins into their biologically active forms (Seidah, Chretien et al. 1994). This enzyme is furthermore involved in the production of a soluble form of the hemochromatosis protein HJV (Silvestri, Pagani et al. 2008), an antagonist involved in bone morphogenetic protein (BMP)-mediated activation of hepcidin (Babitt, Huang et al. 2006), which is an essential regulator of iron homeostasis. It has been shown that furin transcription is modulated by cellular iron levels and by hypoxia (Silvestri, Pagani et al. 2008, McMahon, Grondin et al. 2005); therefore excess iron decreases furin protein levels, and consequently interferes thereby with the production of soluble HJV. On the contrary, iron deficiency or hypoxia upregulates furin activity, in that way increasing the production of HJV, and blocking hepcidin activation (Silvestri, Pagani et al. 2008).

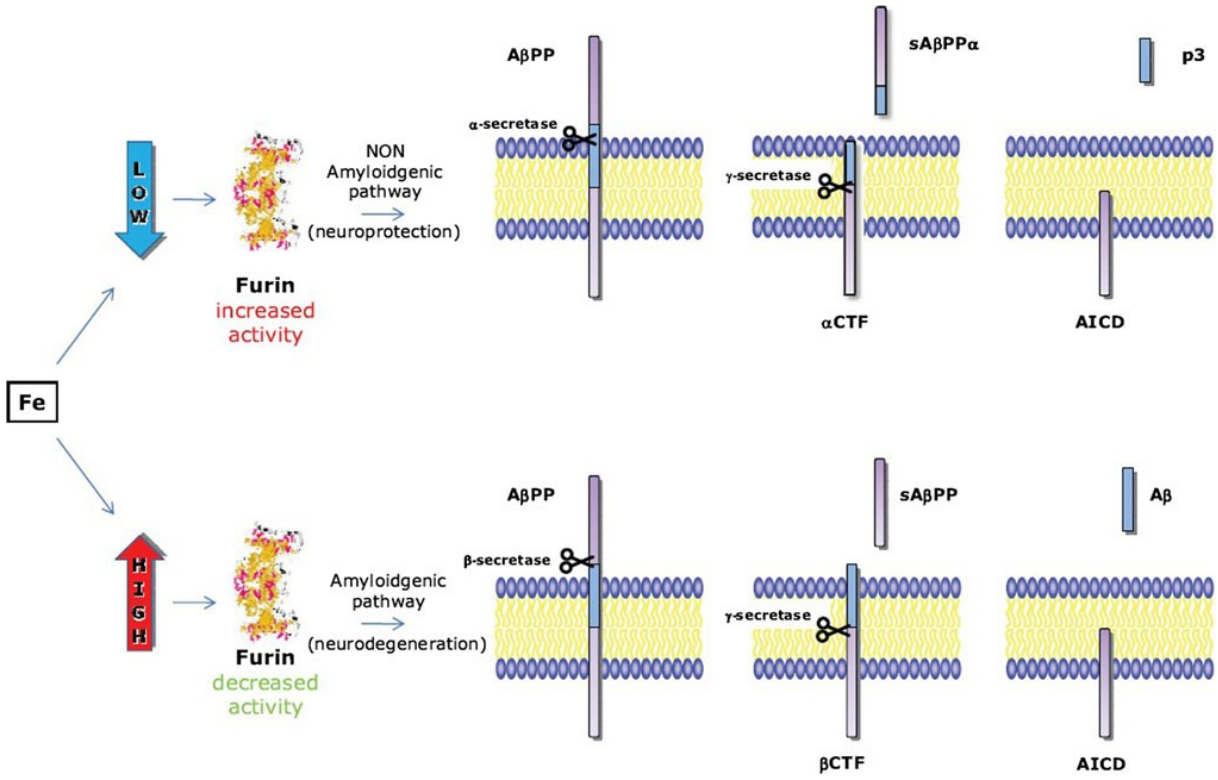

Figure 11. Furin activity and the fate of AβPP cleavage by α- and β-secretases. It is proposed (Silvestri, Camaschella 2008) that low cellular iron levels increase furin activity, stimulating the nonamyloidogenic pathway. In this process furin activates the α-secretase that cleaves AβPP within the Aβ domain generating the extracellular soluble sAβPPα and the membrane bound α-carboxy terminal fragment (αCTF). αCTF is subsequently cleaved by the γ-secretase to produce the soluble fragment p3 and the AβPP intracellular domain (AICD). Thereby Aβ production is prevented resulting in neuroprotection. On the contrary, high cellular iron levels reduce furin activity and may activate the amyloidogenic pathway (adapted from (Altamura, Muckenthaler 2009).

Based on these mechanisms, it has been hypothesized that iron regulation of furin may play a role in AD (Silvestri, Camaschella 2008). A possible pathway could be that increased brain iron levels downregulate furin protein levels, thereby impairing the ability of α-secretase to generate the neuroprotective sAβPPα fragment, in that way activating the amyloidogenic pathway, leading to Aβ production and finally to neurodegeneration (Crichton, Dexter et al. 2011, Silvestri, Pagani et al. 2008). Additionally, it has been proposed (Mueller 2005) that the iron-dependent production of ROS could shift the IRP1 to its IRE-binding form, as a result of that increasing cellular iron uptake via the transferrin receptor, creating a vicious circle which would gradually increase the intracellular iron content, further downregulating furin and shifting the secretase equilibrium in favor of Aβ production. Interestingly, a study (Hwang, Kim et al. 2006) observed that furin mRNA levels in the brains of AD

patients and the AD animal model, Tg2576 mice, were significantly lower than those in controls. Additional results showed that the injection of furin-adenovirus into Tg2576 mouse brains noticeably increased α-secretase activity and reduced Aβ production in affected brain regions.

A further connection between iron metabolism and AD is based on the observation that AβPP expression is iron controlled, as for ferritin, APP levels increase in the presence of iron and decrease by supplementing an iron chelator in neuroblastoma cells (Rogers, Randall et al. 2002). A functional IRE in the 5´-UTR of the AβPP mRNA has been identified. In iron deficient conditions, the AβPP type-II IRE binds IRPs with much low affinity as comparing to normal iron level conditions and leads to reduced AβPP expression (Rogers, Randall et al. 2002). Increased APP formation along with inhibition of a-secretase activity would favor Aβ deposition (Crichton, Dexter et al. 2011).

Another interesting link between iron and AD is demonstrated by the genetic analysis of postmortem brains of 50 AD patients (Coon, Siegel et al. 2006). In this study a significant allelic association between two SNPs (single nucleotide polymorphism) located in the IREB2 gene coding for the IRP2 protein and the AD phenotype were detected. Although it is clear that the number of the study samples needs to be extended, it displays a possible connection between alterations in IRP2 and the heritable risk for the pathogenesis of AD (Coon, Siegel et al. 2006).

Certain brain regions of AD patients such as the hippocampus and the amygdale show signs of increased oxidative stress by demonstrating elevated activities of antioxidant proteins such as glutathione reductase, glutathione peroxidase, superoxide dismutase, and catalase (Zemlan, Thienhaus et al. 1989, Pappolla, Omar et al. 1992).

The occurring oxidative stress may be the result of redox-active iron that is strongly related to the Aβ and NFT deposits in postmortem AD human hippocampal tissues (Altamura, Muckenthaler 2009). Unexpectedly, iron-dependent ROS generation activates IRP1 activity (Pantopoulos, Hentze 1998), thereby increasing iron uptake through TfR1 and decreasing intracellular iron storage capabilities through ferritin-H and L chain. As a result, IRP1 activation by oxidative stress will increase free intracellular iron levels and therefore raising oxidative stress in the cell.

In 1996 Feder and collaborators (Feder, Gnirke et al. 1996) discovered that mutations in HFE explain most cases of hereditary hemochromatosis, as a consequence many studies attempted to discover a connection between systemic iron overload in HFE-associated hereditary hemochromatosis and AD (Berlin, Chong et al. 2004, Candore, Licastro et al. 2003, Combarros, Garcia-Roman et al. 2003, Moalem, Percy et al. 2000, Pulliam, Jennings et al. 2003, Sampietro, Caputo et al. 2001, Robson, Lehmann et al. 2004, Lleo, Blesa et al. 2002). In this context the two most frequent mutations in the HFE gene (C282Y and H63D) with an allele frequency in Caucasians of 5.1% and 15.1%, respectively (Phatak, Ryan et al. 2002), were analyzed. Specifically, homozygous C282Y mutations cause severe iron overload via the disruption of a disulfide bridge in the HFE α3 domain that causes a loss of β-2 microglobulin binding and a lack of HFE cell surface expression (Feder, Tsuchihashi et al. 1997). The HFE protein is present in blood vessels in the brain and the choroid plexus, and is expressed by cells related to neuritic plaques (Connor, Milward et al. 2001). This protein is also expressed by reactive astrocytes and neurons in the brains of AD patients (Zecca, Youdim et al. 2004a). The pattern of neuronal staining for HFE in AD could show that HFE is mediated by stress, and neurons that stain for tau are also HFE-positive (Lee, Connor 2005). Due to the presence of the HFE mutation in AD that result in iron imbalances in the brain thereby contributing to AD, and its prevalence it is suggested that the HFE mutation could represent an important risk factor for AD (Moalem, Percy et al. 2000). Nevertheless, results from epidemiological studies are highly controversial as a number of independent studies were not able to recognize C282Y and H63D mutations as a risk factor for AD (Lleo, Blesa et al. 2002). Further investigations however, identified important connections between C282Y and H63D mutations and AD including a study of HFE mutations in 26 patients with familial AD. In this study HFE mutations were overrepresented in males and underrepresented in females therefore concluding a protective role in women (Moalem, Percy et al. 2000). Moreover, in a further investigation conventional AD patients and non-demented patients with AD-like pathology were examined, showing increased lipid peroxidation in patients with homozygous or compound heterozygous HFE mutations and an important connection to disease in both cases compared to non-demented controls without pathology (Pulliam, Jennings et al. 2003). More research including analysis of 107 patients with sporadic late-onset AD

proposed that the frequency of the H63D mutation was highest in the patients younger than 70 years at the time of disease onset as in comparison to patients over 80 (Sampietro, Caputo et al. 2001).
A further group of studies investigated combined mutations in the HFE gene and additional alleles previously described as AD risk factors, such as the apolipoprotein E ε4 (APOEε4) allele (Saunders, Strittmatter et al. 1993). Carriers of this allele show accelerated cognitive decline as a result of the allele's cumulative impact on the Aβ and NFT biochemical pathways (Mahley, Huang et al. 2007). Combarros et al. analyzed 328 patients with sporadic AD and their results showed that the H63D HFE mutant allele together with the ApoEε4 allele significantly reduces age of onset of AD compared to ApoEε4 carriers alone (Combarros, Garcia-Roman et al. 2003). A further study (Robson, Lehmann et al. 2004) revealed that the combination of HFE mutations and an alternative allele of transferrin (subtype C2) results in a five-fold increased risk for AD. Transferrin is an extremely polymorphic gene with over 30 different allelic variants, whereas a mutation at aminoacid residue 570 (P570S) characterizes transferrin C2 (Lee, Ho et al. 1999). In the past, this mutated transferrin variant was identified with increased frequency in AD patients (Namekata, Imagawa et al. 1997, Zambenedetti, De Bellis et al. 2003), nevertheless, the functional consequences of this mutation need to be further explored (Altamura, Muckenthaler 2009).
Explanations for discrepancies within epidemiological studies on systemic iron overload and the development and progression of AD may include methodological variations in recruitment techniques, participants' stage of dementia, or the measurement of cognition in AD patients (Altamura, Muckenthaler 2009). Furthermore, these discrepancies could reflect on the low clinical penetrance of C282Y homozygous hereditary hemochromatosis in that systemic iron overload may be greatly variable within individuals with HFE mutations (Rossi, Jeffrey 2004).

1.6.3. Parkinson´s disease

PD, which is caused by a selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNPC) (DeLong 1990, German, Schlusselberg et al. 1983, Lozano, Lang et al. 1998), is the second most widespread neurodegenerative

disease after AD affecting about 1% of the population older than 60. In contrast to AD, which affects memory and behavior centers in the brain, PD is described by progressive loss of control over voluntary movement (Crichton, Dexter et al. 2011). The disease was first described in 1817 as “Shaking Palsy” by the English physician James Parkinson (Altamura, Muckenthaler 2009).

The characteristic symptoms of PD (bradykinesia, rigidity, tremor, and loss of balance) develop as a consequence of further loss of 50–70% of the approximately 450,000 dopamine producing cells. Furthermore, PD typically progresses with the loss of 10% of these mid-brain located neurons per year (Lozano, Lang et al. 1998). Even though the etiology of PD is unidentified, mutations have been recognized in the parkin, PINK1, DJ-1 and α- synuclein genes, respectively, which are responsible for rare forms of familial PD (Kitada, Asakawa et al. 1998, Kruger, Kuhn et al. 1998, Polymeropoulos, Lavedan et al. 1997).

The results from various studies have confirmed increasing iron amounts in the substantia nigra (SN) of most severe cases of PD (Dexter, Wells et al. 1987, Riederer, Sofic et al. 1989, Hirsch, Brandel et al. 1991); on the other hand, there still exists controversy about the stage during disease progression at which nigral iron changes take place. Nonetheless, it is generally accepted that total nigral iron levels increase in PD, probably causing nigrostriatal dopamine neuron degeneration as a consequence of its ability to generate ROS and cause lipid peroxidation (Youdim, Ben-Shachar et al. 1991, Jomova, Vondrakova et al. 2010).

Since iron has the ability to catalyze the formation of free radicals, it is most likely that its accumulation contributes to the progression of events leading to neuronal death such as inhibition of complex I of the mitochondrial electron transport chain. Further comprehension of these events derives from studies using the parkinsonian toxin 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) (Langston, Ballard et al. 1983, Schapira, Cooper et al. 1990, Schapira, Gegg 2011). Potent complex I inhibitors such as MPTP or 6-hydroxydopamine (6-OHDA) lead to neuronal death which can be prevented by the pharmacologic or genetic chelation of iron (Kaur, Yantiri et al. 2003, Shachar, Kahana et al. 2004, Youdim, Stephenson et al. 2004, Youdim, Buccafusco 2005, Zheng, Youdim et al. 2010) or by dysfunction of the iron transporter DMT1 (Salazar, Mena et al. 2008). Recent data (Gomez, Aguirre et al. 2011) revealed that low amounts of MPP+, the active metabolite of MPTP, causes

neuritic tree collapse without loss of cell viability in mesencephalic dopaminergic neurons. Prevention of this collapse was effectively succeeded by providing fewer iron amounts or by adding antioxidants.

Further, it has been demonstrated (Chinopoulos, Adam-Vizi 2001, Zhang, Marcillat et al. 1990) that ROS adversely affect complex I activity, moreover an initial inhibition of complex I could probably create a positive loop between ROS generation and further complex I inhibition. Therefore, it can be logically assumed that a vicious cycle of iron accumulation, complex I dysfunction and ROS rise may lead to uncontrolled oxidative damage, and finally culminating in cell death (Nunez, Urrutia et al. 2012).

Early post-mortem studies observed lower GSH levels in degenerating SN of Parkinson's disease patients (Perry, Godin et al. 1982, Sofic, Riederer et al. 1988, Sian, Dexter et al. 1994), suggesting that GSH reduction may has significant effects on neurodegenerative processes. It is debatable whether GSH reduction is an early occurrence during the progression of the disease or a consequence of increased oxidative stress produced by ROS rise and iron accumulation. Chinta et al. demonstrated that chronic submaximal inhibition of GSH synthesis in N27 dopaminergic cells produces 50% inhibition of mitochondrial electron transport chain complex I without inducing cell death (Chinta, Andersen 2006). Hence, a decrease in GSH levels by itself could hinder mitochondrial function. Consequently, inhibition of complex I by reduced GSH levels causes increased electron leakage from the electron transport chain, ROS rise and further iron accumulation. There is still no answer about which of the three processes, reduced GSH levels, inhibition of complex I activity or iron accumulation, start the oxidative spiral, nonetheless, if one of them occurs the other two will follow (Nunez, Urrutia et al. 2012).

A typical pathological feature of PD and a small family of other neurodegenerative disorders including neurodegeneration with brain iron accumulation (NBIA) and diffuse Lewy body disease are intracellular, eosinophilic proteinaceous aggregates within dopaminergic neurons, axons and synapses of the substantia nigra called Lewy bodies. Lewy bodies are composed of granular material in their cores and surrounding 10nm wide radiating filaments and they consist mostly of α-synuclein (Goedert 2001). As previously mentioned, excessive iron is linked to promote the aggregation of α-synuclein, which has now been demonstrated in many studies (Golts, Snyder et al. 2002, Hashimoto, Hsu et al. 1999, Munch, Luth et al. 2000,

Ostrerova-Golts, Petrucelli et al. 2000). Further, it has been publicized that the removal of free iron with the iron chelator desferrioxamine can block α-synuclein aggregation (Hashimoto, Hsu et al. 1999, Sangchot, Sharma et al. 2002). **Figure 12** displays the role of iron in PD.

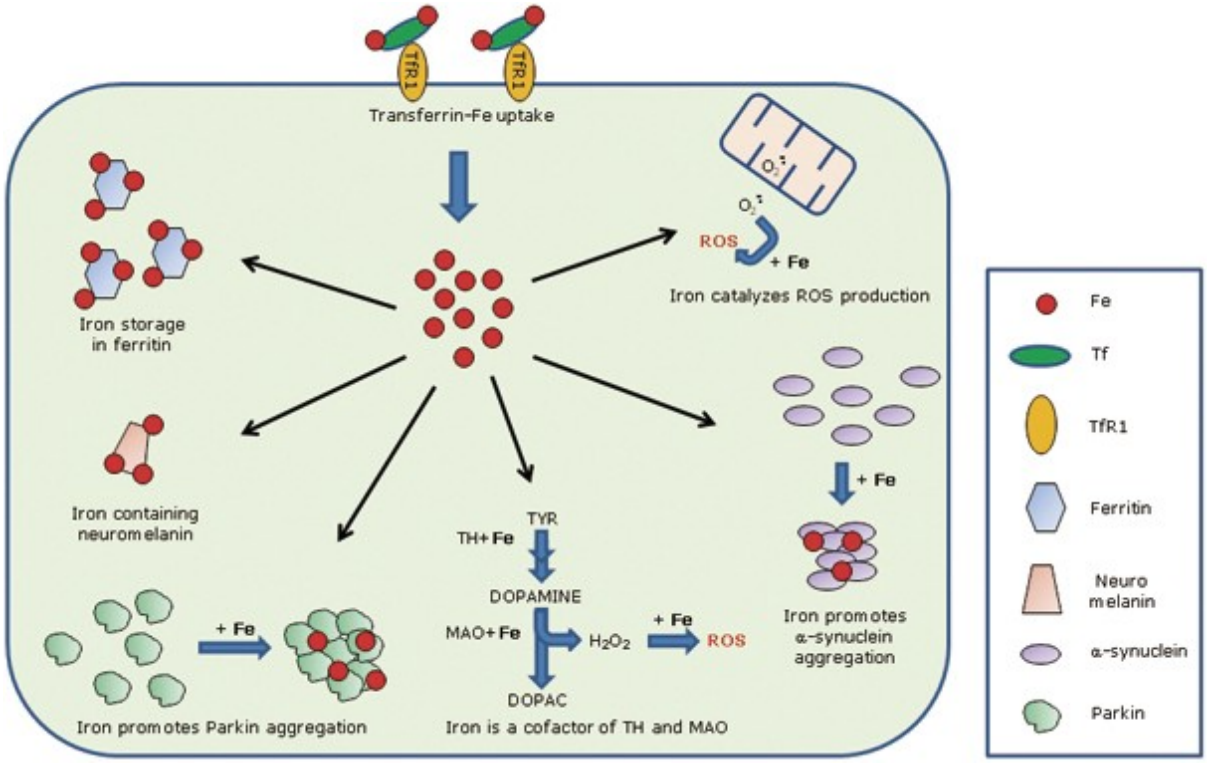

Figure 12. The role of iron in Parkinson's disease. Within the brain interstitial fluid, iron is transported bound to transferrin (Tf) that is absorbed by neuronal cells using transferrin receptor (TfR1) mediated endocytosis. Increased iron levels are in the neurons of the substantia nigra in PD. Excess iron will be stored in ferritin, but ferritin levels are inappropriately low in PD. If the cells capacity for storing iron is exceeded, free iron will accumulate which causes toxicity. Neuromelanin, which is produced in dopaminergic neurons, binds free iron and is also reduced in PD. Iron promotes conformational modifications within Parkin and α-synuclein, which lead to their aggregation. Iron is an essential cofactor of tyrosine hydroxylase (TH) that plays a role in the biosynthesis of dopamine and of monoamine oxidase (MAO), an enzyme which is necessary for dopamine metabolism. Hydrogen peroxide is created by this reaction and it can lead to the conversion of reactive oxygen species (ROS) by the iron-catalyzed Fenton reaction. Superoxide anion O2 is generated during mitochondrial respiration and iron catalyzes its conversion into ROS (adapted from (Altamura, Muckenthaler 2009).

It is still not completely clarified what the biological function of synuclein is but, since it localizes in the cytosol of the presynaptic terminal nearby synaptic vesicles, it has been proposed that it contributes in the modulation of neurotransmitter release (Iwai,

Masliah et al. 1995, Liu, Ninan et al. 2004). A study (Ostrerova-Golts, Petrucelli et al. 2000) demonstrated that BE-M17 neuroblastoma cells overexpressing wild-type or mutated α-synuclein cause more aggregates, when stimulated by iron or free radical generators, like dopamine or hydrogen peroxide. Further results from this study showed that α-synuclein overexpressing cells indicate up to a four-fold increase in vulnerability to toxicity induced by iron. The authors interpreted their results in a way that α-synuclein may act together with iron and dopamine to promote formation of Lewy body pathology and cell death in PD. Hashimoto and colleagues (Hashimoto, Takeda et al. 1999) point out that ferrous iron has to act together with hydrogen peroxide to enhance α-synuclein aggregation, but this process can be prevented by the iron chelator desferrioxamine. A hypothetical IRE within the 5'UTR of the α-synuclein mRNA has been identified, which may thereby lead to define a role for iron in the regulation of α-synuclein expression; nevertheless, the functionality of this IRE sequence needs to be further investigated (Friedlich, Tanzi et al. 2007).

A number of studies (Takanashi, Mochizuki et al. 2001, Castellani, Siedlak et al. 2000, Arawaka, Saito et al. 1998) have shown that iron accumulates within Lewy bodies in the brains of PD patients and thereby probably supporting additional neuronal damage. The aggregation of α-synuclein consequently encourages an accumulation of redox-active iron within the cytosol (Golts, Snyder et al. 2002, Hashimoto, Hsu et al. 1999, Munch, Luth et al. 2000, Ostrerova-Golts, Petrucelli et al. 2000, Hashimoto, Takeda et al. 1999), and further this iron acts as a catalyst for free radical production by Fenton chemistry.

Usually, iron is stored within ferritin clusters that are non-toxic, and therefore not causing oxidative damage (Torti, Torti 2002), but a relative shortage of ferritin and accumulation of ferrous iron can promote oxidative stress and oxidative damage in the dopaminergic neurons of the SN. In line with this concept, Castellani and colleagues (Castellani, Siedlak et al. 2000) displayed that excess iron present in the Lewy bodies in the SNPC of PD patients exists in a redox-active state, and therefore capable to induce oxidative stress. In addition, various neurochemical studies demonstrate strong evidence for oxidative stress in the SN in PD (Connor, Snyder et al. 1995, Mandel, Grunblatt et al. 2003, Turnbull, Tabner et al. 2001, Friedman, Galazka-Friedman 2001, Halliwell 2001, Kienzl, Jellinger et al. 1999, Jenner 1998, Owen, Schapira et al. 1997, Owen, Schapira et al. 1996, Gutteridge 1994).

Beside these investigations, also animal studies further confirm the toxicity of iron for the neurons of the SN (Gerlach, Riederer 1996, He, Thong et al. 1996, Hirsch, Faucheux 1998, Mochizuki, Imai et al. 1994). Local iron application in the SN has been linked to induce neurodegeneration of striatal dopaminergic transmission, as well as the depletion of the striatal dopamine content and neuronal loss in the nigrostriatal system. The occurrence of increased lipid peroxidation confirmed the participation of oxidative stress (Ben-Shachar, Riederer et al. 1991, Lin, Yang et al. 1998, Rauhala, Lin et al. 1998, Sengstock, Olanow et al. 1994). In addition, the intracerebroventricular injection of the iron chelator desferrioxamine has been demonstrated to slow down 6-hydroxydopamine-induced degeneration of nigrostriatal neurons (Ben-Shachar, Riederer et al. 1991).

Consistent with the suggested role of iron in neuronal damage in PD, elevated free iron and reduced ferritin levels have been noted in the SN in postmortem brain tissues from PD patients (Connor, Snyder et al. 1995, Dexter, Carayon et al. 1990, Mann, Cooper et al. 1994). Griffiths et al. (Griffiths, Dobson et al. 1999) observed that in the neurons of PD patients, ferritin is more heavily iron-loaded compared with controls, indicating that inadequate ferritin amounts are expressed to manage the iron load. The neuroprotective role of ferritin is best shown in the neurodegenerative disorder “neuroferritinopathy” (Curtis, Fey et al. 2001), in which insertion of an adenine within the region that encodes the C-terminus of the ferritin L-chain have an effect on the basal ganglia and is linked to intense iron accumulation.

In 2002, Faucheux and colleagues analyzed H- and L-ferritin subunit levels and IRP binding activities in the SN of a group of parkinsonian patients who showed a major reduction in the number of nigral melanized neurons and elevated iron amounts. Their results revealed that iron accumulation was not coupled with higher ferritin subunit concentrations, and further, the lack of ferritin upregulation is connected with persistent IRP-1 activity which is the main form of IRP in the human mesencephalon (Faucheux, Martin et al. 2002). Generally, IRP1 represses ferritin synthesis and IRP1 activity should usually decline when free iron levels rise, hence, persistent IRP1 activity may explain the relative lack of protective ferritin in neurons of the SN in PD (Altamura, Muckenthaler 2009).

Data from an animal study revealed that transgenic mice overexpressing H-ferritin show a reduction of oxidative stress markers in the neurons of the SN when injected

with the neurotoxin MPTP, thereby confirming the protective role of H-ferritin in the brain (Kaur, Yantiri et al. 2003). On the contrary, the observation that elevated brain ferritin levels resulting from IRP2- deficiency correlate with neurodegeneration was interpreted such that increased ferritin levels may cause ‘functional iron deficiency’ in the brain that supports neuronal damage (LaVaute, Smith et al. 2001).There is obviously a need for systematic analysis of brain ferritin levels to define the role of ferritin in neurodegeneration (Altamura, Muckenthaler 2009).

Beside H-ferritin, metal ions in the brain can also be sequestered by neuromelanin (NM), which is, as previously mentioned, a granular dark pigment produced by catecholaminergic neurons of the SN and the locus coeruleus (Gotz, Double et al. 2004). It is known that NM, present in the SN, is detected after the first year of life and increases with age (Zecca, Stroppolo et al. 2004b). Generally, under physiological conditions neuromelanin is saturated with iron about 50%, suggestive of its protective role against oxidative stress by sequestering redox-active iron (Shima, Sarna et al. 1997, Zareba, Bober et al. 1995). Therefore it is of interest that within the SN of PD patients, NM concentrations are reduced by half (Zecca, Fariello et al. 2002), and the correlating redox activity is significantly increased. For that reason it is proposed that the amount of NM in PD is inadequate to compensate for growing iron load in the PD brain and, as a result, free radicals may arise leading to neuronal toxicity (Faucheux, Martin et al. 2003). Hence, NM should probably be regarded as a neuroprotective molecule that buffers elevated iron levels in SN neurons in spite of unchanged ferritin levels. When the buffering capacities of NM are exceeded, it may turn into a source of free radicals (Altamura, Muckenthaler 2009).

Additionally, Faucheux observed an overexpression of lactoferrin receptors, which are able to bind iron reversibly, on neurons and microvessels in regions of neuronal degeneration in PD-affected brain tissue, proposing a potential connection to iron overload in affected brain regions (Faucheux, Hirsch 1998).

Summarizing the above, all these mechanisms are potential contributors to disturbances in iron homeostasis and metabolism in PD occurring at various levels, including iron uptake, storage, intracellular metabolism, release, and posttranscriptional control (Berg, Gerlach et al. 2001).

1.6.4. Multiple Sclerosis

Multiple sclerosis (MS) is a neuroinflammatory disease in which oligodendrocytes and their product myelin are attacked by mononuclear cells. Immunocytochemical and histochemical staining methods displayed high concentrations of ferritin (Connor, Boeshore et al. 1994) and iron in oligodendrocytes (LeVine 1991). In conditions of stress such as hypoxia, oligodendrocytes are able to increase their ferritin synthesis (Qi, Jamindar et al. 1995). Ferritin has the ability to release iron, thereby providing redox-active iron which can contribute to cellular injury by its oxidative processes (Borg 1993). Craelius and colleagues (Craelius, Migdal et al. 1982) observed positive iron reactions in sections surrounding demyelinated plaques in autopsy specimens from five patients with multiple sclerosis. The authors hypothesized that these positive reactions found within blood vessels of gray matter near the demyelination area could also be caused by extravasated blood. In disagreement with the observations of this study (Craelius, Migdal et al. 1982), Walton and Kaufmann (Walton, Kaufmann 1984) were not able to detect iron within or around areas of demyelination in their microscopic examination of autopsy material from patients with multiple sclerosis. Nonetheless, further studies found increased ferric iron content in the putamen and thalamus in an MS brain at autopsy (Drayer, Burger et al. 1987), iron deposits in macrophages and microglia in postmortem MS brain tissue (LeVine 1997), and Mehindate et al. (Mehindate, Sahlas et al. 2001) found an upregulation of heme oxygenase-1 in MS spinal cord astrocytes, proposing a disturbed iron metabolism in patients with MS. A further study, using the animal model of MS, experimental autoimmune encephalomyelitis (EAE), revealed that motor deficits seen in EAE mice can be improved by suppression of heme oxygenase activity (Chakrabarty, Emerson et al. 2003). Beside these findings, iron deposits have also been detected inside macrophages and astrocytes and in extracellular CNS areas in EAE mice compared to control mice (Forge, Pedchenko et al. 1998).
Further data investigating the role of iron in MS also derive from *in vivo* human MRI studies (Bakshi, Dmochowski et al. 2001, Bakshi, Shaikh et al. 2000, Bakshi, Benedict et al. 2002, Bermel, Puli et al. 2005, Drayer, Burger et al. 1987, Grimaud, Millar et al. 1995). The Bakshi group (Bakshi, Dmochowski et al. 2001, Bakshi, Shaikh et al. 2000, Bakshi, Benedict et al. 2002) observed cortical and subcortical gray matter hypointensities on T2-weighted MR images in MS patients, suggesting

that these pathologic iron amounts are a surrogate marker of the destructive disease process. A more recent study (Khalil, Langkammer et al. 2011) quantitatively assessed regional brain iron levels using R2* relaxometry in 113 patients with MS, concluding that elevated brain iron levels are likely an epiphenomenon of MS pathology and not a cause of the disease.
Nonetheless, more data deriving from longitudinal studies as well as from different stages of MS patients are definitely needed to further define the role of brain iron in MS (Khalil, Langkammer et al. 2011).

1.6.5. Huntington`s disease

Huntington´s disease (HD), a neurodegenerative disorder, is typically described by progressive motor, cognitive, and psychiatric decline. On average, onset of symptoms is in middle-age (30 and 50 years old), but the disease manifestation can vary between infancy and advanced age. The source of origin of this neurodegenerative disorder derives from a dominant glutamine expansion (CAG repeat coding) within the N-terminal of the huntingtin protein which initiates events causing neuronal loss primarily within the striatum and cerebral cortex (Batista-Nascimento, Pimentel et al. 2012). Further, it has been reported that this huntingtin protein is iron responsive (Hilditch-Maguire, Trettel et al. 2000). Full-length huntingtin is large (~ 350kD), but it is the smaller N-terminal fragments that are responsible for disease progression (Graham, Deng et al. 2006). These smaller fragments have abnormal interactions with themselves and other biomolecules that cause the molecular characteristics of HD including aggregates, transcriptional repression (DiFiglia, Sapp et al. 1997), oxidative damage, and metabolic dysfunction (Browne, Bowling et al. 1997).
In HD patients, increased iron levels have mainly been detected in the basal ganglia, specifically, in the striata and the globus pallidus (Bartzokis, Cummings et al. 1999). Furthermore, it has been observed that ferritin-iron levels are increased in striata of early clinical HD patients as measured by magnetic resonance imaging (Bartzokis, Tishler 2000). Iron amounts rise in early stage of HD and continue to rise with age, which indicates that iron may be involved in the progression of the disease; nonetheless, the mechanisms involved in this process are not yet clarified (Batista-

Nascimento, Pimentel et al. 2012). Even though, both AD and PD are hallmarked by iron accumulation, the iron regulation patterns seem to differ from HD (Bartzokis, Cummings et al. 1999). For example, PD is characterized by increased iron amounts in the substantia nigra, whereas this observation has not been noticed in HD. Bartzokis and colleagues (Bartzokis, Tishler 2000) suggest that increased iron amounts in HD may not be a byproduct of the disease itself as they seem to occur at the onset of the disease, and therefore representing a putative risk factor.

1.6.6. Friedreich ataxia

Friedreich ataxia (FA) is an autosomal recessive neurodegenerative disorder and the most frequent of all heritable human ataxias. FA is characterized by degenerative atrophy of the spinocerebellar and pyramidal tracts, dorsal columns of the spinal cord, dorsal root ganglia, and, to a minor extent, the cerebellum and medulla (Pandolfo 2008). These degenerations result in clinical manifestations such as progressive dysarthria, gait and limb ataxia (incoordination), areflexia, loss of proprioception and vibration sense, pes cavus, diabetes mellitus and hypertrophic cardiomyopathy. In general, these clinical manifestations arise before age 25 and pathological conditions like hypertrophic cardiomyopathy may lead to secondary neurological morbidities such as thromboembolic cerebral infarction which could be responsible for premature death in these patients (Gille, Reichmann 2011). MRI and histochemical stain studies has demonstrated that iron accumulates in the brain, specifically in the cerebellar dentate nuclei, and in the myocardium of FA patients (Babady, Carelle et al. 2007, Lamarche, Cote et al. 1980, Waldvogel, van Gelderen et al. 1999).
Friedreich ataxia is in most cases (>95%) caused by a GAA nucleotide repeat expansion in the first intron of the frataxin gene leading to reduced frataxin mRNA expression (Campuzano, Montermini et al. 1996). Frataxin is a mitochondrial protein and consequently highly expressed in mitochondria-rich tissues including brain, heart, as well as skeletal muscle where it probably assists in the assembly or export of iron–sulfur clusters from mitochondria, heme biosynthesis and mitochondrial iron storage (Gille, Reichmann 2011, Becker, Richardson 2001).

The reduced generation of frataxin in FA patients results in intramitochondrial iron trapping, and defective aerobic respiration (Becker, Richardson 2001).

1.6.7. Genetic factors and disturbed iron regulation in the brain

In recent years, progresses in neurogenetics, such as powerful autozygosity mapping, have lead to the identification of a number of genes that are related to disturbed brain iron metabolism and could probably lead to syndromes of neurodegeneration with brain iron accumulation (NBIA) (Schneider, Dusek et al. 2013). The two most common syndromes that account for most cases of NBIA, are the neuroaxonal dystrophies pantothenate kinase-associated neurodegeneration (PKAN, formerly established as Hallervorden-Spatz disease, HSS,), presently classified as NBIA type 1 and PLA2G6-associated neurodegeneration (PLAN), classified as NBIA type 2. Further NBIA syndromes include ferritin light polypeptide mutation in neuroferritinopathy and ceruloplasmin mutation in aceruloplasminemia (Ke, Ming Qian 2003). Additionally, mutations in the HFE gene are thought to be associated with the onset of AD and PD (Ke, Qian 2007). **Figure 13** displays some gene mutations and their underlying neurodegenerative syndromes.

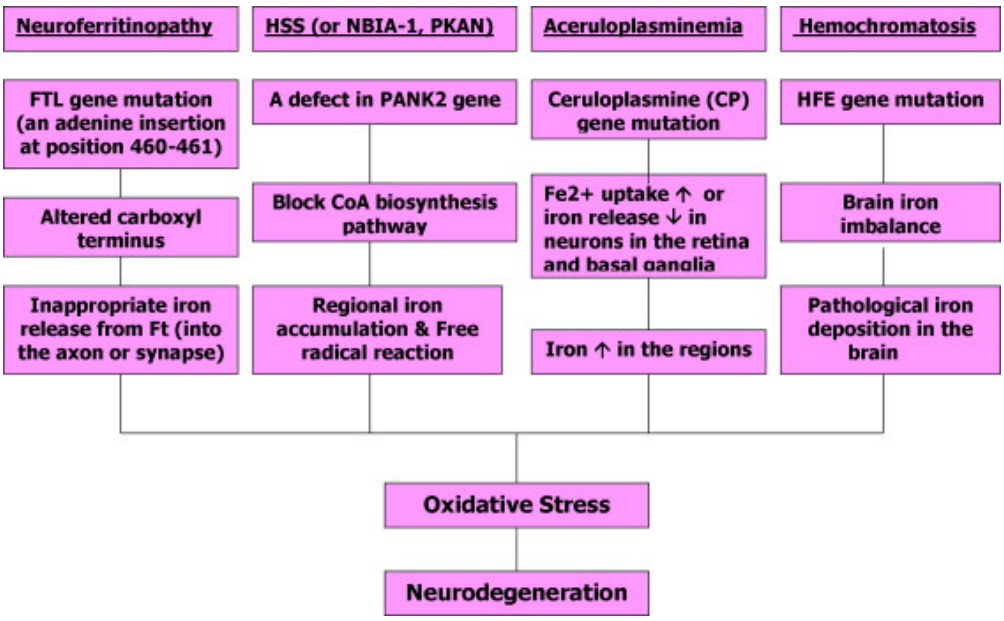

Figure 13. Genetic factors leading to brain iron misregulation in the development of neurodegenerative disorders. Abbreviations: FTL, ferritin light polypeptide; HFE, hemochromatosis protein; HSS, Hallervorden–Spatz syndrome; NBIA-1, neurodegeneration with brain-iron accumulation-1; PKAN, pantothenate kinase associated neurodegeneration;

PANK2, a novel pantothenate kinase; Ft, ferritin; CoA, coenzyme A. Adapted from (Ke, Qian 2007).

1.6.7.1. NBIA type 1–PKAN (former Hallervorden-Spatz disease)

Type I neurodegeneration with brain iron accumulation (NBIA-1) or PKAN, formerly known as Hallervorden-Spatz disease, is a rare (worldwide prevalence 1: 1.000.000), genetically determined neurodegenerative disorder described by extrapyramidal dysfunction and mental deterioration (Schneider, Dusek et al. 2013). The disease generally develops during the first two decades of life, classically before the age of 6 years in almost 90% (Hayflick 2006, Hayflick, Westaway et al. 2003), commonly presenting gait difficulty as initial symptom (Hayflick, Westaway et al. 2003). Further symptoms include pyramidal (spasticity and hyperreflexia) and extrapyramidal features such as dystonia, Parkinsonism, and chorea. Also a number of neuropsychiatric features (e.g. cognitive decline) as well as oculomotor abnormalities are seen (Schneider, Dusek et al. 2013).
Additionally, gene-proven cases with late-onset (atypical) PKAN (in the 20s and 30s) have been reported (Hayflick, Westaway et al. 2003, Antonini, Goldwurm et al. 2006, Aggarwal, Schneider et al. 2010). The phenotype may be to some extent atypical and compared to the classical form, motor symptoms tend to be less severe.
Typically, PKAN has a progressive course and affected patients usually become wheelchair-bound within a few years (Schneider, Dusek et al. 2013).
The disease is characterized by iron accumulation mostly in the globus pallidus and the pars reticularis of the substantia nigra, which is shown as brown-pigmented iron deposits (Zhou, Westaway et al. 2001). A further characteristic feature of NBIA-1 is the “eye-of-the-tiger” sign, a centrally located high signal in the globus pallidus seen in MRI contrast studies (Hayflick 2003, Ponka 2002). The genetic basis for the disease, which is caused by a defective pantothenate kinase gene (PANK2), has been identified by Zhou and colleagues (Zhou, Westaway et al. 2001). Generally, PANK2 is most highly expressed in neurons of the cortex, globus pallidus, nucleus basalis of Meynert, and pontine nuclei. Pantothenate kinase is an important regulatory enzyme that is involved in the biosynthesis of coenzyme A, catalyzing the cytosolic phosphorylation of pantothenate (vitamin B5), N-pantothenoyl-cysteine, and pantetheine. The product of the reaction is phosphopantothenate (Zhou, Westaway

et al. 2001). Coenzyme A is indispensable for fatty acid synthesis; therefore dysfunction of PANK2 probably causes derangement in lipid metabolism. It is known that PANK2 is for the most part targeted to mitochondria; consequently, its mutation may also cause dysfunction of cellular energy metabolism (Lin, Beal 2006).
It is assumed that the defective PANK2 blocks a metabolic pathway in the brain causing cysteine accumulation, which binds iron and consequently leads to iron accumulation resulting in oxidative stress. While it is known that PANK2 is not directly involved in iron metabolism, it is suggested that its absence may contribute to brain iron accumulation, leading to neuronal death by a free radical pathway (Rouault 2001, Zhou, Westaway et al. 2001, Hayflick 2003, Hayflick 2006).
Further, Poli and colleagues (Poli, Derosas et al. 2010) proposed that the variation of ferroportin expression mediated by PANK2 might be associated with brain iron accumulation.
Nevertheless, at present, the physiological functions of PANK2 have not been completely clarified and more research, other than its catalytic activity, is definitely required (Ke, Qian 2007).

1.6.7.2. NBIA Type 2 – PLA2G6 associated neurodegeneration (PLAN)

The second most common NBIA syndrome is PLAN resulting from PLA2G6 gene mutations (NBIA type 2). As in the case of PKAN, it appears that there is an age-dependent phenotype, whereas early-onset cases have infantile neuroaxonal dystrophy (INAD). INAD is described by progressive motor and mental retardation, marked truncal hypotonia, cerebellar ataxia, pyramidal signs, and early visual disturbances caused by optic atrophy (Morgan, Westaway et al. 2006).
Characteristically, neuroimaging displays cerebellar atrophy in early stages of INAD, other than in late-onset disease. While half of INAD patients do not show signs of iron accumulation early in the disease course (Morgan, Westaway et al. 2006), they frequently develop hypointensities of the globus pallidus reflecting iron, noted on T2, T2* and proton density –weighted images (Kurian, Morgan et al. 2008). Interestingly, the signal abnormality is different from the “eye of the tiger” sign in PKAN patients, showing no central hyperintensity. It has been noticed that iron accumulation in the

substantia nigra occurs in some atypical cases of NBIA-type 2 (Gregory, Polster et al. 2009, Paisan-Ruiz, Li et al. 2012). Nevertheless, in contrast to PKAN, iron accumulation is not a universal feature of PLAN; further the majority of late-onset cases do not show signs of iron deposits and MRI may even be totally normal. Other patients could show cortical atrophy or white matter changes. Therefore, not all forms of PLA2G6-related neurodegeneration belong to the group of NBIA; however there exists “neuroradiological variability” (Paisan-Ruiz, Bhatia et al. 2009).
It has been suggested that the mutation in the PLA2G6 gene is related to altered lipid composition of the plasma membrane, vesicles, or endosomes. As a result, these alterations could affect proteins and processes usually involved in regulating the movement of membranes within axons and dendrites, consequently resulting in the accumulation of membranes in distal axons, finally culminating in progressive neurological impairment (Malik, Turk et al. 2008, Adibhatla, Hatcher 2010, Adibhatla, Hatcher 2008). Comparing PKAN to PLAN, pathologically, in PLAN-associated neurodegeneration alterations are more widely distributed throughout the CNS (Vinters, Farrell et al. 1998). Pathological patterns, described early (Cowen, Olmstead 1963), included cerebellar atrophy and sclerosis, accumulation of lipid and gliosis in the striatum, as well as degeneration of the optic pathway and of some of the long tracts in the brain-stem and spinal cord. Recent mouse model (Malik, Turk et al. 2008) and human brain (Paisan-Ruiz, Li et al. 2012, Gregory, Westaway et al. 2008) studies found widespread alpha-synuclein-positive Lewy pathologies. Further findings include accumulation of hyperphosphorylated tau and neurofibrillary tangles, again contrary to PKAN (Paisan-Ruiz, Li et al. 2012).

1.6.7.3. Neuroferritinopathy

In 2001, Curtis and colleagues (Curtis, Fey et al. 2001) were the first to describe neuroferritinopathy, a dominantly inherited, late-onset disease (around age 40) of the basal ganglia that is characterized by extrapyramidal features comparable to those of Huntington's and Parkinson's diseases. It has been observed that patients with this disorder show abnormal aggregates of iron and ferritin in the brain and low serum ferritin concentrations resulting from a mutation in the gene for ferritin light polypeptide (FTL). Further, brain histopathology demonstrates widespread reddish

discoloration of the basal ganglia and findings in the globus pallidus include numerous roughly spherical inclusions up to 50 μm in diameter that contain iron; the majority of these inclusions contain ferritin. Beside these histopathological findings, a MRI study observed also cystic changes in the basal ganglia and bilateral pallidal necrosis, as well as iron accumulation in the caudate, globus pallidus, putamen, SN, and red nuclei (McNeill, Birchall et al. 2008). A further recent study found in the asymptomatic phase of the disorder hypointense signals, suggestive of early iron accumulation, as seen in three gene mutation carriers (Keogh, Jonas et al. 2012). The authors of this study also observed an increasing severity of T2* abnormalities with age, concluding that iron accumulation in neuroferritinopathy actually starts in childhood but the disorder remains asymptomatic until midlife. Consequently, appropriate timing of the chelating therapy which should be optimally initiated in childhood would be necessary in order to prevent further iron accumulation (Keogh, Jonas et al. 2012).

Generally, iron and ferritin are present in several areas of the forebrain and cerebellum in all people, nevertheless, the number and size of iron or ferritin elements in the brains of patients with neuroferritinopathy significantly exceed those detected in healthy elderly people. However, the distribution pattern for iron in neuroferritinopathy is similar to that of normal aging human brains (Curtis, Fey et al. 2001).

The mutation in the FTL gene in patients with neuroferritinopathy causes an alteration of the carboxyl-terminal residues of the gene product (the light subunit of the ferritin protein), leading to a disturbed structure and function of ferritin, causing the spontaneous release of highly toxic iron into the axon or synapse, and consequently resulting in oxidative stress and neuronal cell death (Curtis, Fey et al. 2001, Rouault 2001). Favoring conditions such as the highly polarized nature of the neuron as well as the axonal trafficking of iron-loaded ferritin could represent a possible explanation why major pathologies are restricted to the nervous system (Rouault 2001). Whereas a later study (Vidal, Ghetti et al. 2004) found also ferritin accumulation in extraneural tissue such as skin, muscle, kidney and liver. A further study (Mancuso, Davidzon et al. 2005) also found extracerebral pathologies such as hepatic iron deposits.

Due to the abnormality in ferritin it is strongly suggested that iron plays a main role in the pathogenesis of this neurodegenerative disorder and a potential treating possibility for neuroferritinopathy includes iron chelators such as desferrioxamines (Curtis, Fey et al. 2001).

1.6.7.4. Aceruloplasminemia

Aceruloplasminemia is an autosomal recessive disease, caused by a mutation in the ceruloplasmin gene on chromosome 3q in which more than 40 mutations have been displayed (Schneider, Dusek et al. 2013). The clinical presentation includes retinal degeneration, diabetes mellitus, and neurological symptoms such as ataxia, blepharospasm, dystonia, tremor, Parkinsonism, and chorea along with cognitive dysfunction and dementia (Miyajima 2003, Miyajima, Nishimura et al. 1987, Miyajima, Takahashi et al. 2002, Hatanaka, Okano et al. 2003). A literature review disclosed an average age at diagnosis of 51, varying between 16 to 71 years (McNeill, Pandolfo et al. 2008).

As previously mentioned, the encoded protein ceruloplasmin plays an important role in the mobilization of iron from tissues through its ferroxidase activity and carries 95% of the plasma copper. Clinical and pathologic studies on patients with aceruloplasminemia observed a noticeable accumulation of iron in affected parenchymal tissues along with a lack of circulating serum ceruloplasmin (Gitlin 1998, Grisoli, Piperno et al. 2005). The most prominent and unique feature of the disorder is the progressive neurodegeneration of the retina and basal ganglia in combination with excessive iron accumulation in these tissues and cells (Harris, Klomp et al. 1998). However, excessive iron accumulation is not only restricted to the brain (basal ganglia, thalami, dentate nuclei and cerebral and cerebellar cortices) but also within the retina, pancreas and liver. Interestingly, the severe cortical involvement has not been documented in other NBIAs and most likely underlies the high prevalence of cognitive dysfunction (Schneider, Dusek et al. 2013). Several autopsy studies observed a mild degree of cortical atrophy, large iron deposits in basal ganglia, thalami, dentate nuclei and cerebral cortices mainly in the perivascular spaces localized typically to terminal astrocytic processes and malformed astrocytes with swollen, oxidatively damaged astrocytic foot processes appearing as globular

structures (Gonzalez-Cuyar, Perry et al. 2008, Kaneko, Hineno et al. 2012, Oide, Yoshida et al. 2006). Consequently, the results of these studies lead to the conclusion that astrocytes, which are required for brain iron uptake, detoxification and further trafficking, are mostly affected in this disorder. Further findings in autopsied brains (Miyajima, Takahashi et al. 2003) include reduced activity of mitochondrial respiratory chain complexes I and IV and increased markers of lipid peroxidation. In general, it is strongly indicated that enhanced oxidative stress resulting from redox active iron is a main cause of neurodegeneration in aceruloplasminemia (Kaneko, Hineno et al. 2012, Oide, Yoshida et al. 2006, Yoshida, Kaneko et al. 2000). Further, neuronal cell death could be to some extent secondary to the deficit of protective function usually provided by astrocytes.
Even though aceruloplasminemia is regarded autosomal recessive, slightly elevated iron deposits in liver and basal ganglia as well as neurologic symptoms have been seen also in heterozygotes (McNeill, Birchall et al. 2008, Mariani, Arosio et al. 2004, Daimon, Susa et al. 2000, Miyajima, Kono et al. 2001). Diagnostically in homozygotes, ceruloplasmin is classically not detectable in the serum, moreover copper and iron serum levels are low, whereas ferritin levels are increased 3–40-fold (McNeill, Pandolfo et al. 2008). Additionally, Miyajima and colleagues observed hypometabolism in the basal ganglia and the thalamus by using FDG-PET (fludeoxyglucose- positron emission tomography) (Miyajima, Takahashi et al. 2003). Further findings by Miyajima and colleagues (Miyajima, Takahashi et al. 1997) revealed that treatment with the iron chelator desferrioxamine is able to reduce brain iron stores, therefore preventing the progression of neurological symptoms, and decrease plasma lipid peroxidation in this disease. As it was proposed in the treatment of neuroferritinopathy (Keogh, Jonas et al. 2012), also in aceruloplasminemia an early chelation therapy is recommended to reduce central nervous system iron accumulation and to prevent or improve neurological symptoms related to neurodegeneration. Again, these observations confirm that iron misregulation caused by ceruloplasmin gene mutation is a main source in the pathogenesis of this disorder (Miyajima, Takahashi et al. 1997).
Table 1 displays the comparison of aceruloplasminemia and neuroferritinopathy.

	Aceruloplasminemia	Neuroferritinopathy
Gene	Ceruloplasmin gene	Ferritin light chain gene
Pattern on Inheritance	Autosomal recessive	Autosomal dominant
Presentation	Third decade: diabetes, anemia Fifth decade: neurologic	Third through sixth decade
Defect	Brain iron recycling	Brain iron storage
Pathogenesis	Brain iron accumulation Systemic iron accumulation in all	Brain iron accumulation Systemic iron accumulation in some
Clinical	Diabetes, anemia, dementia Dystonia, dysarthria	Dementia, dystonia, dysarthria
Pathology	Iron accumulation in astrocytes Neuronal loss	Iron accumulation in astrocytes Neuronal loss

Table 1. The comparison of aceruloplasminemia and neuroferritinopathy. Modified from (Madsen, Gitlin 2007) and reproduced from (Schneider, Dusek et al. 2013).

For further NBIA syndromes and related gene mutations please see (Schneider, Dusek et al. 2013).

1.6.7.5. Hemochromatosis in association with AD and PD

As previously mentioned, the HFE protein is an important membrane protein which is involved in cellular iron uptake (Arredondo, Munoz et al. 2001, Griffiths, Sly et al. 2001, Beutler 2006). It is known that mutations in the HFE gene are frequently related to type 1 hereditary hemochromatosis, which results in iron overload disease in homozygotic and some heterozygotic individuals. In the past, the brain was assumed to be unaffected by the peripheral iron accumulation observed in hereditary hemochromatosis. Yet, recent observations have led to reconsideration of this view (Ke, Qian 2007). Several studies propose that carrying an HFE mutation is a risk factor for AD or PD (Lee, Connor 2005, Connor, Lee 2006) and other neurodegenerative disorders (Demarquay, Setiey et al. 2000, Wang, Lee et al. 2004). HFE mutations and the association with AD have been previously discussed, please see section 1.6.2. Alzheimer´s disease.

Over the years several case reports of PD and Parkinsonism associated with hemochromatosis have been published (Nielsen, Jensen et al. 1995, Buchanan, Silburn et al. 2002, Costello, Walsh et al. 2004, Papanikolaou, Samuels et al. 2004,

Thomas, Jankovic 2004). A study by Dekker and colleagues (Dekker, Giesbergen et al. 2003) investigated the role of mutations in the HFE gene in PD and other Parkinsonism (non-PD Parkinsonism) in two population-based series. Their results indicate that the HEF mutation (C282Y) increases the risk of PD and other Parkinsonism (non-PD Parkinsonism). However, a review by Russo and colleagues (Russo, Edwards et al. 2004) about movement disorders in connection with the HFE mutation as well as data regarding brain iron accumulation came to the conclusion that movement disorders are unusual in connection with hereditary hemochromatosis, and that such patients should be extensively examined for another cause for their movement disorder.
At this time, it is not possible to identify whether HFE mutations in fact contribute to pathological brain iron accumulation and earlier onset of symptoms in AD and PD patients. Hence, more research is definitely needed (Ke, Qian 2007).

1.7. Magnetic resonance imaging and brain iron

Over the years, progressions have been made in the development of noninvasive, *in vivo* techniques for assessing brain iron accumulation using magnetic resonance imaging (MRI). Therefore, several different techniques have developed to investigate brain iron deposition in normal aging and in neurodegenerative diseases, which will be discussed here.

1.7.1. The effect of iron on MRI signal

In general, mobile protons in the tissues ultimately define the signal which will be detected by MRI. In the brain, these mobile protons are nearly completely present in the solvent water molecules as H_2O. Variations among brain regions of the density of solvent water and the two relaxation times, longitudinal (T1) and transverse (T2), of the associated protons contribute to the image contrast in the resulting MR image. Magnetic substances as well as paramagnetic substances in the brain such as iron and a number of other metals lead to the shortening (reduction) of T2 relaxation time, which will be reflected as hypointensity on T2-weighted images, and shortening of T1 relaxation time appearing as hyperintensity on T1-weighted images (Schenck 2003).

It is of great importance to distinguish between two forms of iron, both detectable in the human body: 1) heme iron, such as hemoglobin and 2) nonheme iron (Schenck, Zimmerman 2004, Schenck 2003, Haacke, Cheng et al. 2005). In the brain, nonheme iron is stored in a mineralized form named ferrihydrite and could be possibly shielded from water by a protein shell when present in its most frequent molecular forms, ferritin or hemosiderin, or less frequently transferrin. It is suggested that paramagnetic ions cause an effect on MRI contrast by two probable mechanisms: the outer and the inner sphere mechanism. The effect of both ferritin and hemosiderin on MRI contrast arises through the outer sphere mechanism (Schenck, Zimmerman 2004, Schenck 2003). In this mechanism iron-containing cores of ferritin and hemosiderin are magnetized by the applied field and an induced microscopic dipole magnetic field surrounds every core (Koenig, Brown 1984, Koenig, Kellar 1995). Newer models have also been published postulating a direct contact between water molecules and the core surface (Brooks 2002, Gossuin, Roch et al. 2002). The induced microscopic magnetic fields surrounding the iron deposits cause local field inhomogeneities (LFI) (additional proton spin dephasing) resulting in hypointensities on T2-weighted images with insignificant or no effect on T1 relaxation time. Beside affecting the transverse relaxation time, T2, iron also affects the corresponding rate, R2, that describes the time during which the proton spins interact with each other (spin-spin interactions) and the signal in the transverse plane is lost (Sullivan, Adalsteinsson et al. 2009).The rate of signal loss and the consequential image hypointensity is defined through the size and magnetization of the particles and their density, the water diffusion constant and the details of the pulse sequence (Schenck, Zimmerman 2004).

1.7.2. Different MRI methods for detecting brain iron deposits

In the mid-1980s, after clinical MR imaging was introduced for a while, it was quickly recognized that T2-weighted images using long echo times, demonstrated hypointensities in the similar areas typically related to high iron accumulation (Drayer, Burger et al. 1986). The first studies were done at 1.5 T, however after a little while it was noticed that this contrast increased rapidly by using higher field strength

magnets (Schenck, Mueller et al. 1989; 9, Kell 2009, Kell 2009, Schenck 1995, Bizzi, Brooks et al. 1990).

Numerous pulse sequences, each one with a different form of sensitivity, have been utilized to identify iron deposits in tissues including repeated single spin echo (Schenck 1995), dual spin echo, fast spin echo (FSE) (Allkemper, Schwindt et al. 2004), multiple spin echo (CPMG) with iron sensitivity measured by varying the inter-echo spacing (Ye, Martin et al. 1996, Ye, Martin et al. 1996), gradient echo, and hybrid forms such as gradient-echo slice excitation profile imaging (GESEPI) (Yang, Smith et al. 1999, Yang, Williams et al. 1998), partially refocused interleaved multiple echo (PRIME) (Ordidge, Gorell et al. 1994, Miszkiel, Paley et al. 1997) and gradient echo sampling of free induction decay and echo (GESFIDE) (Gelman, Gorell et al. 1999). Further approaches include the measurement of phase shifts close to brain iron deposits (Reichenbach, Venkatesan et al. 1997, Ogg, Langston et al. 1999).

The most relevant relaxometry metrics for the detection of brain iron are the relaxation rates R2, R2*, and R2′ deriving from T2, T2* and T2′ relaxation time decay curves. R2, R2*, R2′ are the inverse of T2, T2*, and T2′ (T2=1/R2, T2*=1/R2*, R2′=1/T2′) and increase when non-heme iron is present (Stankiewicz, Panter et al. 2007). The higher the iron amounts, the greater are the hypointensities on T2- and T2*-weighted images (Schenck 2003). The simple dual spin echo offers the most uncomplicated measurement of T2 and the dual gradient echo does the same for T2* (Schenck, Zimmerman 2004). R2* is the fastest of the three transverse decay rates, because the decay takes place with no benefit of partial signal restoration when radiofrequency (RF) refocusing pulses are used to solicit spin echoes after excitation. R2 is a slower transverse relaxation rate measured from a series of spin echoes obtained at different echo times and created with RF refocusing pulses in contrast to gradient reversals (Brass, Chen et al. 2006). RF pulse refocusing is able, for instance, to compensate for the proton dephasing arising near microscopic field gradients associated with iron deposits (Zywicke, van Gelderen et al. 2002).

There exists a number of *in vitro* (Vymazal, Brooks et al. 1996, Chen, Hardy et al. 1989) and *in vivo* (Gelman, Gorell et al. 1999, Metafratzi, Argyropoulou et al. 2001, Schenck 1995, Vymazal, Righini et al. 1999, Hardy, Gash et al. 2005, Graham, Paley et al. 2000) studies that have revealed a strong correlation between R2 and iron deposits as defined by histological studies of brain gray matter. A weaker correlation

is available for white matter iron, most likely caused by the influence of tissue water on R2 (Chen, Hardy et al. 1989, Whittall, MacKay et al. 1997). Consequently, R2 alone is not a specific or sensitive indicator of iron levels when pathological tissue is involved. The confounding effect of tissue water content on R2 impedes the ability to assess iron deposits in neurologic diseases in connection with a wide range of pathologic processes that increase water content (e.g. inflammation, gliosis, edema, and axonal/neuronal loss) (Chen, Hardy et al. 1989, Whittall, MacKay et al. 1997, Haacke, Cheng et al. 2005).

The more complex pulse sequence R2′ is the difference between R2* and R2, and arises from the transverse relaxation mechanisms that reflect the reversible signal losses related to LFI (Haacke, Cheng et al. 2005, Hikita, Abe et al. 2005, Gelman, Gorell et al. 1999). R2′ is seen as probably most sensitive to iron accumulation, in particular ferritin (Vymazal, Brooks et al. 1995). The benefit of R2′ is that it is dependent of the susceptibility effect related to iron and it is independent of other parameters which have an effect on R2 (Brass, Chen et al. 2006). Nevertheless it has been observed that R2′ is hindered by artifacts associated with field inhomogeneity (Haacke, Cheng et al. 2005, Hikita, Abe et al. 2005). A publication by Gelman and colleagues (Gelman, Gorell et al. 1999) investigated the correlation of R2 and R2′ at 3T with iron deposits in SN, red nucleus, globus pallidus, putamen, caudate, prefrontal cortex, and frontal white matter in humans. The authors observed that R2′ demonstrated a better correlation with tissue iron levels than R2. Similar findings were also seen in other studies (Ordidge, Gorell et al. 1994, Gorell, Ordidge et al. 1995). On the other hand, a further investigation (Hikita, Abe et al. 2005) measured R2′ and R2 in the basal ganglia of 13 healthy volunteers and correlated these relaxometry metrics with iron content obtained from historical postmortem data (Hallgren, Sourander 1958). A linear correlation was recognized between R2 and iron accumulation and between R2′ and iron accumulation; however the correlation between MRI and iron amounts in the gray matter was higher for R2 than R2′. The R2′ measurements were influenced by field inhomogeneities associated with the skull and therefore less sensitive to interregional differences (e.g. iron in the cortex vs. putamen).

Over the course of several years, mapping of the R2* relaxation rate constant has been recognized as one of the most potential methods for measuring brain iron

accumulation in normal aging as well as in neurodegenerative disorders (Aquino, Bizzi et al. 2009, Peran, Hagberg et al. 2007, Yao, Bagnato et al. 2012, Khalil, Langkammer et al. 2011, Khalil, Enzinger et al. 2009). R2* is assessed directly from multiecho gradient-echo MR images obtained at different echo times (Wild, Martin et al. 2002). This easily accessible spoiled gradient echo sequence provides artifact-free high resolution images of the total brain in clinical suitable time, which is a great advantage of this method, when comparing R2* mapping with other well-recognized MRI methods for assessing brain iron accumulation (Ropele, de Graaf et al. 2011). Further, R2* mapping has been verified in correlation studies against chemical determination of iron levels (Langkammer, Krebs et al. 2010, Wood, Enriquez et al. 2005), revealing a linear relationship between R2* measurements and iron concentration over the complete physiological range of concentrations. In addition, Kuhlpeter and colleagues (Kuhlpeter, Dahnke et al. 2007) demonstrated in experimental studies that quantitative R2* maps were linearly correlated with the cellular iron load. Additional data deriving from initial animal studies indicate that mapping of R2* represents an indirect measure of iron (Zywicke, van Gelderen et al. 2002, Gilissen, Jacobs et al. 1999). All these data confirm that quantitative R2* maps allow a noninvasive estimation of iron accumulation in the brain. While tissue iron accumulation is the main determinant of R2*, also other factors may affect this MR parameter such as magnetic field inhomogeneities arising from tissue-tissue and air-tissue interfaces (Aquino, Bizzi et al. 2009, Reichenbach, Venkatesan et al. 1997). Nevertheless, the measurement of R2* is simple and for that reason free of accumulation of errors that come up from aggressive multistep postprocessing of noisy data (Aquino, Bizzi et al. 2009). Further, Langkammer and colleagues (Langkammer, Krebs et al. 2010) demonstrated that R2* is more sensitive than R2 to variations in brain iron concentration and that R2* can determine variations in iron concentration in white matter. A recent multicenter study (Ropele, Wattjes et al. 2013) investigated the inter-scanner and inter-subject variability of R2* mapping in healthy subjects and concluded that R2* is a robust and reproducible measure in a multicenter setting in the application of a standardized MRI protocol. Additionally, according to a latest publication (Langkammer, Ropele et al. 2014) that compared available MR techniques for quantitative iron mapping in the brain, R2* -based imaging was revealed as the best validated technique for the detection of brain iron.

Other methods for measuring iron content include the field dependent rate increase (FDRI) method which has extensively been used by Bartzokis and colleagues (Bartzokis, Sultzer et al. 2000, Bartzokis, Tishler 2000, Bartzokis, Sultzer et al. 1994, Bartzokis, Cummings et al. 1999, Bartzokis, Aravagiri et al. 1993, Bartzokis, Mintz et al. 1994, Bartzokis, Beckson et al. 1997). This method is based on the increase in the transverse relaxation rate (R2=1/T2) with increasing field strength, as a result the difference between tissue R2 at two different MRI field strengths (e.g. 1.5 T and 3 T) is measured. It is stated that the difference is a specific measure of ferritin content (Bartzokis, Aravagiri et al. 1993). The Bartzokis group has consistently observed higher FDRI values in the basal ganglia region of AD and PD brains, compared with healthy controls (Bartzokis, Sultzer et al. 1994, Bartzokis, Cummings et al. 1999, Bartzokis, Sultzer et al. 2000). Nevertheless, the disadvantages of the FDRI method include that R2 is calculated from two contiguous, 3-mm thick slices, which affords low spatial resolution and does not exclude partial volumes effects (Bartzokis, Cummings et al. 1999, Bartzokis, Tishler et al. 2007, Bartzokis, Tishler et al. 2004) and this method requires two different MR scanners (which the majority of radiological centers do not have) and further has an acquisition time that lasts about one hour (Peran, Hagberg et al. 2007).

Susceptibility weighted imaging (SWI) is another possibility to detect brain iron. This technique uses local phase differences to map iron, resulting from the effects of ferritin iron and other paramagnetic substances on the product of signal amplitude and a filtered version of the signal phase at a given field strength and echo time (Pfefferbaum, Adalsteinsson et al. 2009). An advantage of SWI over FDRI is that SWI does not require scanning at multiple field strengths (Haacke, Ayaz et al. 2007, Haacke, Xu et al. 2004), nevertheless limitations of this method include that SWI is more prone to distortion by air/bone/tissue interfaces and structural topography relative to the magnetic field. Further, both phase and R2 are affected by heme iron (deoxygenated hemoglobin), but the effect on phase is much more than on R2 and can therefore result in confound precise assessment of non-heme iron levels. Certainly, the strong contrast between gray and white matter on gradient echo imaging of cerebral cortex may be caused, at least to some extent, to the greater blood volume of gray matter (Duyn, van Gelderen et al. 2007).

Magnetic field correlation (MFC), developed by Jensen and colleagues (Jensen, Chandra et al. 2006), is a new technique that uses asymmetric spin echoes to quantify brain iron. This technique is based on the influence of MR signal by magnetic field inhomogeneities (caused by spatial variations in magnetic susceptibility). These changes can also be caused by macroscopic structures including cavities, bones, vessels, and microscopic structures for example capillaries, as well as the presence of paramagnetic substances (e.g., metals, contrast agents). MFC imaging derives from a theoretical model of MR relaxation in the occurrence of magnetic field inhomogeneities and uses a non-monoexponential decay curve. According to Jensen and colleagues (Jensen, Chandra et al. 2006) MFC maps show more contrast between the basal ganglia and adjacent tissue than corresponding R2 maps concluding that MFC imaging may be more sensitive than R2 for quantifying brain iron, whereas comparisons with other MR parameters such as R2* or R2′ are lacking. Since MFC grows with the square of applied field strength, it was recommended that high field scanners (e.g., 3T or greater) are most suitable for MFC imaging. However, water diffusion and non-uniform diffusion as seen in edematous tissues influence MFC and may lead to errors. Further limitations include the fact that MFC measures magnetic field inhomogeneities, which can be also caused by any type of magnetic substance such as other transitional metals present in tissue (Jensen, Chandra et al. 2006).

Further methods for assessing brain iron have been proposed such as MR phase imaging (Ogg, Langston et al. 1999), measurement of the newer relaxometry parameter T2 rho (Wheaton, Borthakur et al. 2004), direct saturation imaging (Smith, Bulte et al. 2009), and the lately introduced quantitative susceptibility mapping technique which was shown to be highly sensitive but also specific to cerebral iron deposition (Langkammer, Schweser et al. 2012).

Summarizing the above, there exist a number of different MR techniques that visualize and quantify brain iron, each one with its advantages and limitations. **Table 2** displays some of the current MR methods available for iron mapping in the brain. For more details about measuring iron by using MRI, please see (Haacke, Cheng et al. 2005).

METHOD	ADVANTAGES	LIMITATIONS
Visual rating of T2 and T2* hypointensities	Available from conventional MRI No computational post-processing	Not quantitative Low sensitivity Rater dependent
R2 relaxometry	Sequence is readily available on clinical systems (R2 can be calculated from multi-echo spin-echo sequence) Good sensitivity for iron Robust against susceptibility artifacts	Moderate acquisition speed In multi-slice acquisition, R2 may be affected by magnetization transfer effects
R2* relaxometry	Sequence is readily available on clinical systems (R2* can be calculated from multi-echo gradient echo sequence) Fast 3D whole-brain acquisition (<10 min) High sensitivity for iron	Calcifications cannot be separated from clustered iron deposits
Phase imaging	Images come for free when performing a multi-echo gradient echo sequence High sensitivity for iron Calcifications can be distinguished from iron deposits	Phase unwrapping and filtering needed Head position, tilting and postprocessing impact results Not a linear measure for iron
Susceptibility-weighted imaging	Good sensitivity for iron Calcifications can be distinguished from iron deposits Enhanced contrast	Same as for phase imaging Not quantitative (depends on postprocessing parameters)
Quantitative susceptibility mapping	High sensitivity for iron Linear measure for iron Calcifications can be distinguished from iron deposits	Extensive image post-processing

Table 2. Comparison of current MRI techniques for the visual and quantitative assessment of brain iron. Adapted from (Langkammer, Ropele et al. 2014).

Although many different methods were used to investigate brain iron accumulation over the years, similar findings were observed. There is an age-related increase in non-heme iron levels in subcortical structures such as the nucleus caudatus, putamen, globus pallidus, substantia nigra and red nucleus (Bartzokis, Beckson et al. 1997, Martin, Ye et al. 1998, Ogg, Langston et al. 1999, Thomas, Boyko et al. 1993, Aoki, Okada et al. 1989, Aquino, Bizzi et al. 2009, Bilgic, Pfefferbaum et al. 2012, Pfefferbaum, Adalsteinsson et al. 2009), as well as increased iron accumulation in many neurodegenerative disorders (Bartzokis, Sultzer et al. 2000, Bakshi, Dmochowski et al. 2001, Drayer, Burger et al. 1987, Bartzokis, Tishler 2000, Vymazal, Righini et al. 1999, Gorell, Ordidge et al. 1995, Bartzokis, Cummings et al. 1999). Overall, there is controversy whether increased brain iron deposition is a direct cause of neurodegeneration, a secondary event in a pathophysiological

cascade, or just a nonspecific marker of neurodegeneration (Schneider, Dusek et al. 2013).

1.8. Research question / Hypothesis

As previously mentioned, in the past, MRI has mainly been used to evaluate brain iron accumulation in neuroinflammatory (Drayer, Burger et al. 1987, Bakshi, Benedict et al. 2002, Bermel, Puli et al. 2005) and neurodegenerative diseases (Bartzokis, Tishler 2000, Brass, Chen et al. 2006, Thomas, Boyko et al. 1993). If iron-related oxidative stress precedes the hallmark of neuropathological lesions of neurodegeneration, it is probable that regional iron concentrations in brain tissue correlate to clinical findings at the earliest stages of neurodegenerative disorders. There exist only a small number of authors (Bartzokis, Lu et al. 2011, Pujol, Junque et al. 1992, Sullivan, Adalsteinsson et al. 2009, Rodrigue, Daugherty et al. 2013) that have investigated the association of brain iron and cognition in non-demented individuals. A recent report has suggested an inverse relationship between iron deposits in the basal ganglia and cognition in the general population (Penke, Valdes Hernandez et al. 2012). In addition, a latest publication (Li, Langkammer et al. 2014) explored the correlation between magnetic susceptibility, a surrogate marker of brain iron, of gray matter structures with behavioral measures of motor and cognitive ability in healthy adults, revealing that increased magnetic susceptibility in the globus pallidus and red nuclei was associated with decreasing manual dexterity.

Consequently, the purpose of the present study was to investigate the impact of R2*-based brain iron on cognition in healthy individuals free of history or signs of stroke and dementia.

1.8.1. Main hypothesis

We hypothesized (1) that increased iron concentrations are inversely associated with cognitive performance following region-specific patterns, and (2) that such associations, if any, are mediated by the magnitude of brain atrophy or vascular brain lesions.

2. METHODS

2.1. Participants

The Austrian Stroke Prevention Family Study (ASPS-Fam) is a prospective single-center, community-based study on the cerebral effects of vascular risk factors in the normal aged population of the city of Graz, Austria. ASPS-Fam represents an extension of the Austrian Stroke Prevention Study (ASPS), which was established in 1991 (Schmidt, Lechner et al. 1994, Schmidt, Fazekas et al. 1999). Between 2006 and 2013, study participants of the ASPS and their first grade relatives were invited to enter ASPS-Fam. Inclusion criteria were no history of previous stroke or dementia and a normal neurologic examination. A total of 381 individuals from 169 families were included into the study. The number of members per family ranged from 2 to 6. The entire cohort underwent a thorough diagnostic work-up including clinical history, laboratory evaluation, cognitive testing, and an extended vascular risk factor assessment. All individuals underwent MRI, except for 26 who had contraindications. Thus, voxel- based R2* maps were available in a total of 355 participants. Nineteen participants were excluded because of brain infarcts; therefore the sample for the current study comprises 336 individuals. Dementia was an exclusion criterion and none of the study participants fulfilled the Petersen criteria for amnestic mild cognitive impairment (MCI) (Petersen 2004). However, 7 individuals whose age was between 61 and 77 years had a Mini-Mental State Examination (MMSE) score below 25 with a minimum of 22. One person had a score below 1.5 standard deviations of the mean in the memory domain, but he did not report memory problems. His clinical dementia rating (CDR) score was zero. All individuals with MMSE scores below 25 or memory performance below 1.5 standard deviations of the mean had a low educational level with a maximum of 8 years of schooling.

The study protocol was approved by the ethics committee of the Medical University of Graz, Austria, and written informed consent was obtained from all participants.

2.2. Vascular risk factors

Assessment of vascular risk factors included arterial hypertension, diabetes mellitus, and cardiac disease and was determined based on history and measurements at the examination as previously described (Schmidt, Fazekas et al. 1999). Diagnosis of vascular risk factors relied on the individuals' histories and appropriate laboratory findings. Arterial hypertension was considered present if a participant had a history of arterial hypertension with repeated systolic/diastolic blood pressure readings >140/90 mm Hg or if the readings at examination exceeded this limit. Diabetes mellitus was coded present if a participant was treated for diabetes at the time of the examination or if the fasting blood glucose level at examination exceeded 126mg/dL. Cardiac disease was assumed to be present if there was evidence of cardiac abnormalities known to be a source for cerebral embolism (Kittner, Sharkness et al. 1990), evidence of coronary heart disease according to the Rose questionnaire (ROSE 1962) or appropriate ECG findings (BLACKBURN, KEYS et al. 1960) (Minnesota codes I: 1 to 3; IV: 1 to 3; or V: 1 to 2), or if an individual presented with signs of left ventricular hypertrophy on echocardiogram or ECG (Minnesota codes III: 1; or IV: 1 to 3).

2.3. Neuropsychological testing

A test battery assessing memory, executive function as well as psychomotor speed was administered as described previously (Schmidt, Fazekas et al. 1999). The tests employed have been widely used in the German-speaking area and were always applied in the same order and under same laboratory conditions. The tests chosen represent examples of the cognitive domains memory and learning, executive functions and psychomotor speed. Yet, it is important to note that the three groups of tests cannot be considered statistically independent, and there exists potential overlap of tests across domains.

Intermediate memory recall and learning ability was assessed by the "Bäumler's Lern- und Gedächtnistest" (LGT-3) (Bäumler 1974), a highly demanding, paper-pencil procedure consisting of six subtests. Three subtests (word and digit association tasks, and story recall) screen for verbal memory, and two subtests (trail and design recall) screen for figural memory. The sum of scores from these subtests

and of an image recognition paradigm result in the total learning and memory performance score. The stimulus sets of the word association task (German-Turkish word pairs), the story (facts about construction of a hospital), and design recall (core symbol and frame), and the recognition paradigm (objects) consist of 20 items each. A trail in an abstracted city map serves as the trail recall test. These sets of stimuli were presented to the person being tested for 1 minute. Two minutes were given for learning the 13 items of the digit association task (three-digit telephone numbers and names of extension holders). During a learning phase the six sets of stimuli are subsequently presented to the person being tested. The recall phase starts immediately thereafter and follows the same order. The delay between presentation and recall for a given subtest ranges between 7 and 11 minutes. Executive functions were tested by the Wisconsin Card Sorting Test (Heaton 1981), part B of the Trail Making Test (United States War Department. 1944) and Digit Span Backwards, which is part of the Wechsler Adult Intelligence Scale, revised (Tewes 1991). Adhering to Milner's criteria (Milner B. 1963), the measures computed for the Wisconsin Card Sorting test were categories completed, perseverative errors, and total errors. Psychomotor speed was evaluated by the Purdue Pegboard Test (Tiffin, Asher 1948). To reduce floor and ceiling artifacts and other sources of measurement error, we used summary measures of cognitive function in the analyses rather than the results of individual tests. We formed composite measures of the specific domains of cognitive function. Each summary measure was calculated by converting individual test scores to z-scores within the group and by computing the average of the scores in each cognitive domain.

Global cognitive function was calculated as the mean of all three cognition variables including memory, executive function and psychomotor speed. **Figure 14** displays how the neuropsychological tests were combined into domain scores.

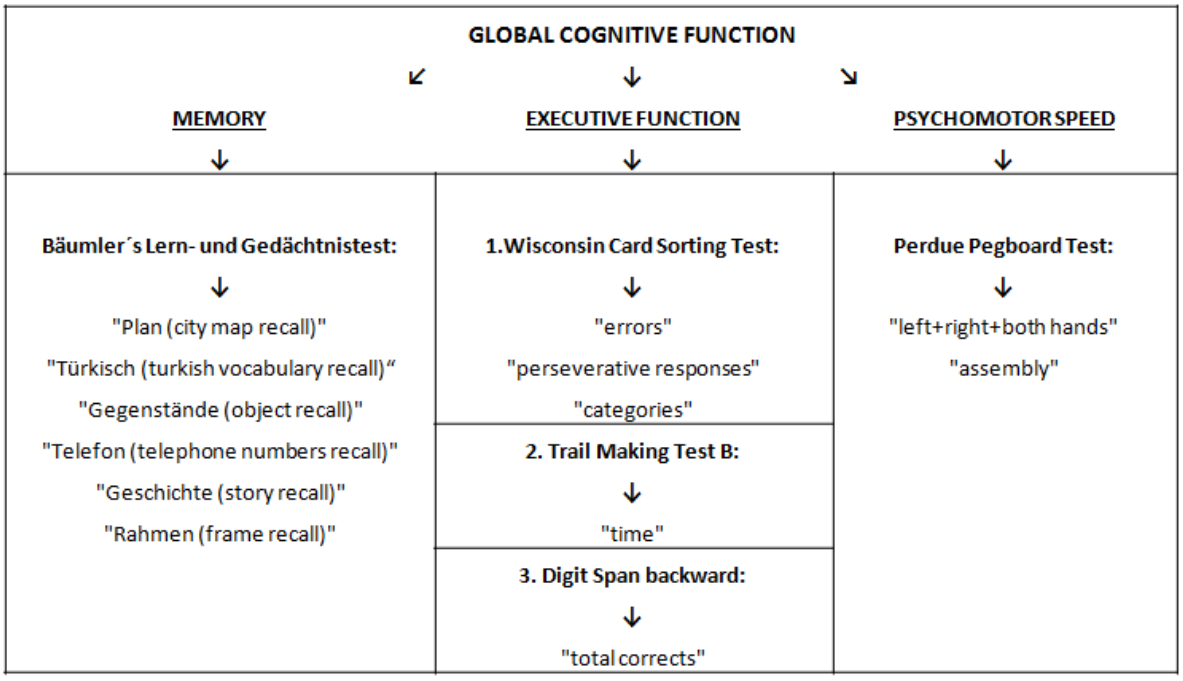

Figure 14. Neuropsychological tests including subtests.

2.4. Magnetic Resonance Imaging

The study participants underwent conventional MR imaging and R2* mapping on a 3T whole-body MR system (TimTrio; Siemens Healthcare, Erlangen, Germany). The conventional protocol included an axial FLAIR sequence (TR=10000ms, TE=70ms, TI=2500ms, number of slices=44, slice thickness=3mm, in-plane resolution=0.86x0.86mm², FOV of 165 x 220 x 132mm3) and a high resolution T1 weighted 3D sequence with magnetization preparation and rapid gradient echo (MPRAGE) with whole brain coverage (TR=1900ms, TE=2.19ms, TI=900ms, flip angle=9°, isotropic resolution of 1 mm) for assessing brain volume and for segmentation. For R2* mapping, a spoiled 3D multi-echo gradient echo sequence (fast low angle shot (FLASH)) was used (FOV of 256 x 256 x 128mm3, matrix = 256 x 256, BW= 190Hz/px, TE of first echo = 4.92ms, echo spacing = 4.92ms, TR=35ms, slice thickness = 2mm, number of slices = 64, number of echoes = 6).

2.5. Visual and volumetric assessment

White matter hyperintensities (WMH) and lacunar infarcts were recorded on FLAIR images as previously described (Schmidt, Fazekas et al. 1999). Lacunes were focal lesions involving the basal ganglia, the internal capsule, the thalamus, the brainstem or the white matter not exceeding a maximum diameter of 20 mm. WMH were outlined using a home written IDL program (Exelis Visual Information Solutions, USA). They were semiautomatically segmented by combined region growing and local thresholding following manual selection. Total WMH volume (mm^3) was calculated from the lesion masks using the program FSLMATHS (FSL, Oxford, www.fmrib.ox.ac.uk). Total brain tissue volume was calculated from the T1weighted MPRAGE images using the fully automated structural image evaluation of FREESURFER (Freesurfer, http://surfer.nmr.mgh.harvard.edu). WMH volume and brain tissue volume were normalized for total intracranial volume (TIV) [nVol=Vol／TIV×100].

2.6. Regional R2* mapping

To determine regional brain iron load, we used R2* mapping technique, which has previously been validated by inductively coupled plasma mass spectrometry in post-mortem studies by our own group (Langkammer, Krebs et al. 2010). The results of these validation studies indicated a correlation coefficient of 0.95 between R2* values and iron concentration throughout the brain. For regional R2* mapping, the gradient-echo (GRE) sequence with 6 echoes and equidistant echo-spacing of 4.92ms was used. R2* values were calculated for deep grey matter including pallidum, putamen, nucleus caudatus and thalamus as well as for total cortex and hippocampus. We used a voxel based monoexponential fit implemented in MATLAB, which is based on an efficient and stable Levenberg-Marquard-Fletcher method (Balda 15 Nov 2007 (Updated 18 Feb 2012)), and considers the noise level measured for each echo. In order to prevent fitting errors influencing the regional analysis, highly noisy voxels, in which the standard deviation of the fit was less than 0.8, were not taken into account. Regional analysis was performed with FSLSTATS, in which R2* maps were evaluated for regions obtained by FREESURFER segmentation. Region masks, originally segmented in the T1-space, were registered to the GRE-space using FSL-

FLIRT (FSL, Oxford, www.fmrib.ox.ac.uk). To avoid partial volume effects introduced by a normal “nearest-neighbour” registration of the masks, we used the “trilinear interpolation” option and a threshold of 90%, which kept significant voxels in the borderline.

2.7. Statistical analysis

Assumptions of normal distribution were tested with the Kolmogorov-Smirnov test. Normally distributed variables are reported as mean +/- standard deviation and non-normally distributed variables as median and interquartile range. Due to the bimodal distribution of age we used tertiles of age (38 years to 60 years; 61 years to 70 years; 71 years to 86 years) in all statistical models. WMH volume had a skewed distribution and therefore the volumes were natural log transformed.
Significant differences in means of normally distributed continuous variables were tested by ANOVA and significant differences of non-normally distributed variables by the Kruskal-Wallis test. Difference in proportions was assessed by X2 statistics.
Regression analyses were used to assess three major sets of hypotheses. First, we tested the association between MRI findings and R2* based iron measurements (hypothesis set 1). Second, we tested the association between iron load and cognition (hypothesis set 2), and third, we determined the association between iron load and cognition mediated by MRI findings (hypothesis set 3).
The association between MRI findings and iron load was assessed in six mixed models, with the MRI variables brain volume, WMH volume and presence of lacunes being the predictor variables, and iron content in each brain region including total cortex, hippocampus, pallidum, putamen, caudate nucleus and thalamus being the dependent variable. In total, 24 comparisons were made.
The association between R2* based iron measurements and cognition was tested in 24 mixed model analyses, with iron load in each of the six brain regions as the predictor variable and each cognitive variable (memory, executive function, psychomotor speed and global cognitive function) as dependent variable.
This association was also tested for verbal and figural memory separately in 12 additional analyses, as well as in each of the three age groups which resulted in 72 comparisons, in addition.

To determine the shape of iron load-related effects in regions significantly associated with cognition, participants were categorized into quartiles according to iron value distribution, and 8 mixed models adjusted for possible confounders with iron quartiles being the explanatory variables and cognitive performance being the dependent variable were calculated. The lowest quartile of iron load served as the reference. A p-value for the linear trend of the iron load-related effect was calculated by including the quartiles as a continuous variable in the mixed models.

To account for the sample relationships in the current family-based study, a random effect was added to each model using a kinship matrix describing the family structure (Newton-Cheh, Eijgelsheim et al. 2009, Suchindran, Vana et al. 2009).

To test the hypothesis that R2*-based iron effects on cognition were mediated by brain atrophy or vascular brain lesions we used simple mediation models for estimating indirect effect size (Preacher, Hayes 2004). Mediation was evaluated in regions significantly associated with cognition for each of the structural brain lesions separately and for each significant cognitive function, resulting in 36 models (two models for each combination of predictor, mediator and independent variable).

We determined whether the influence of R2*-based iron load on cognition is explained by brain volume, WMH volume and presence of lacunar infarcts. Mediator effect sizes and 95% confidence intervals were estimated using a bootstrap-based method developed by Preacher and Hayes (Preacher, Hayes 2008). If the 95% CI of the indirect effect does not contain zero, a significant mediating effect is probable, whereas no mediation is present if zero is included in the 95% CI.

To determine the proportion of the age-related variance in cognition that is accounted for by the R2*-based iron in regions significantly associated with cognition, we used a method developed by Lindenberger and Pötter (Lindenberger, Pötter 1998). The proportion of the variance in cognition that is shared by age and R2* based iron is defined as the shared effect of age and R2* based iron divided by the simple effect of age. The simple age effect is the coefficient of determination (R2) of a model with age as the predictor variable and cognition as the dependent variable. In order to obtain the shared effect of age and R2* based iron, the unique age effect is subtracted from the simple age effect. The unique age effect is the difference in the coefficients of determination of two models with R2* based iron, and R2* based iron and age respectively as independent variables and cognition as dependent variable.

All models (except the models for the determination of the age-related variance) were adjusted for potential confounders to evaluate the independent effect of iron on structural brain abnormalities and cognition. We considered age, sex, education, hypertension, diabetes and cardiac disease as possible confounders. Multicollinearity was assessed between the independent variables of the models using the variance inflation factor (VIF). A VIF value > 10 was considered to be an indicator of multicollinearity. There was no indication for multicollinearity in the models. The mean VIF for predictor variables in our study was 1.53 (range 1.06 – 2.57).
For each regression coefficient ß, the 95% confidence interval and the p-value were determined. A two-sided p-value <0.05 was considered to be statistically significant. Multiple testing correction was performed using the Benjamini-Hochberg false discovery rate (FDR) method (Benjamini, Hochberg 1995). We corrected the p-values of all analyses in hypothesis sets 1 and 2, which are 56 tests in total. Mixed models and kinship matrices were calculated using the coxme (Therneau, 2012) and kinship2 (Therneau, 2013) packages in R (R: A language and Environment for Statistical Computing, Vienna, Austria. www.R-project.org). R was also used to correct the p-values for multiple testing with the p-adjust function.
Multicollinearity check and mediation analyses were performed with SPSS (IBM Statistics for Windows, Version 20, Armonk, New York).

3. RESULTS

Demographics, frequency of vascular risk factors, neuropsychological test results and MRI findings of the total cohort and in tertiles of age are displayed in **table 3**. Higher age was associated with lower educational level, higher frequency of risk factors, worse cognitive performance, greater extent of focal brain lesions and lower brain volume. **Figure 15** gives R2* values as an indirect measure of iron content in various brain regions. As can be seen from this figure, iron load is highly variable depending on brain region. The highest concentrations were observed in the pallidum, followed by the putamen and caudate nucleus. Increased iron content correlated with higher age in putamen (β=2.55; 95%CI 1.12, 3.97; p=0.00044), caudate nucleus (β=1.27; 95%CI 0.24, 2.31; p=0.016) and thalamus (β= -0.57; 95%CI -0.99, -0.15; p=0.0076), but not in pallidum and neocortical structures. Only the association in putamen remained significant after multiple testing adjustment with Benjamini-Hochberg FDR with p=0.01. No association was detected between iron content in any brain region and sex (data not shown). Also, there were no significant associations between R2* values in any of the brain regions and structural MRI changes including WMH volume and brain volume. Only lacunes related to higher R2* values in total cortex after adjustment for multiple testing (β=0.11; 95%CI 0.03, 0.19; p=0.047), but not in other tissue compartments.

	Age (total)	Age category 1 (38-60 years)	Age category 2 (61-70 years)	Age category 3 (71-86 years)	p
Age, years (median, IQR)	67.00 (55-72)				
N (%)	336 (100)	97 (28.9)	123 (36.6)	116 (34.5)	
Women N (%)	204 (60.7)	53 (54.6)	82 (66.7)	69 (59.5)	0.183[a]
Education N (%)					
Primary school N (%)	68 (20.2)	1 (1.0)	38 (30.9)	29 (25.0)	<0.001[a]
Apprenticeship N (%)	142 (42.3)	36 (37.1)	62 (50.4)	44 (37.9)	0.071[a]
High school diploma N (%)	70 (20.8)	30 (30.9)	9 (7.3)	31 (26.7)	<0.001[a]
University degree N (%)	56 (16.7)	30 (30.9)	14 (11.4)	12 (10.3)	<0.001[a]
B. Risk factors					
Hypertension N (%)	212 (63.1)	41 (42.3)	84 (68.3)	87 (75.0)	<0.001[a]
Diabetes N (%)	33 (9.8)	3 (3.1)	15 (12.2)	15 (12.9)	0.030[a]
Cardiac disease N (%)	79 (23.5)	10 (10.3)	29 (23.6)	40 (34.5)	<0.001[a]
C. Neuropsychological testing					
Global cognitive function[1] (range)	-1.75 – 2.21	-1.16 – 2.21	-1.13 – 1.13	-1.75 - 1.22	<0.001*
Memory[1] (range)	-3.17 – 3.50	-0.99 – 3.50	-1.70 – 2.66	-3.17 – 1.74	<0.001*
Executive function[1] (range)	-2.78 – 1.36	-1.17 – 1.36	-1.82 – 0.95	-2.78 – 1.13	<0.001*
Psychomotor speed[1] (range)	-2.39 – 3.14	-2.01 – 3.14	-2.01 – 2.08	-2.39 – 1.73	<0.001*
MMSE (mean, SD)	28.13 (1.47)	28.75 (1.01)	27.99 (1.56)	27.77 (1.55)	<0.001*
MMSE (range)	22 - 30	25 - 30	23 - 30	22 - 30	
D. MRI variables					
Lacunes N (%)	24 (7.1)	2 (2.1)	11 (8.9)	11 (9.5)	0.069[a]
Brain volume normalized for TIV in % mean(SD)	70.89 (3.73)	73.02 (4.37)	70.54 (3.01)	69.46 (2.99)	<0.001*
WMH volume normalized for TIV in % (median, IQR)	0.27 (0.15 – 0.52)	0.16 (0.08 – 0.29)	0.31 (0.17 – 0.53)	0.44 (0.23 – 0.80)	<0.001[b]

Table 3. Demographics, risk factors, neuropsychological test performance and MRI findings of study participants.
Abbreviations: MRI= Magnetic Resonance Imaging; WMH= White Matter Hyperintensities; SD= Standard Deviation; IQR= interquartile range; MMSE= mini mental state examination; normalized in % of TIV= nVol= $\mathrm{Vol}/\mathrm{TIV} \times 100$; nVol= normalized Volume; TIV= total intracranial volume. [a] X2 test, *ANOVA test, [b] Kruskal-Wallis test, [1] Z-values.

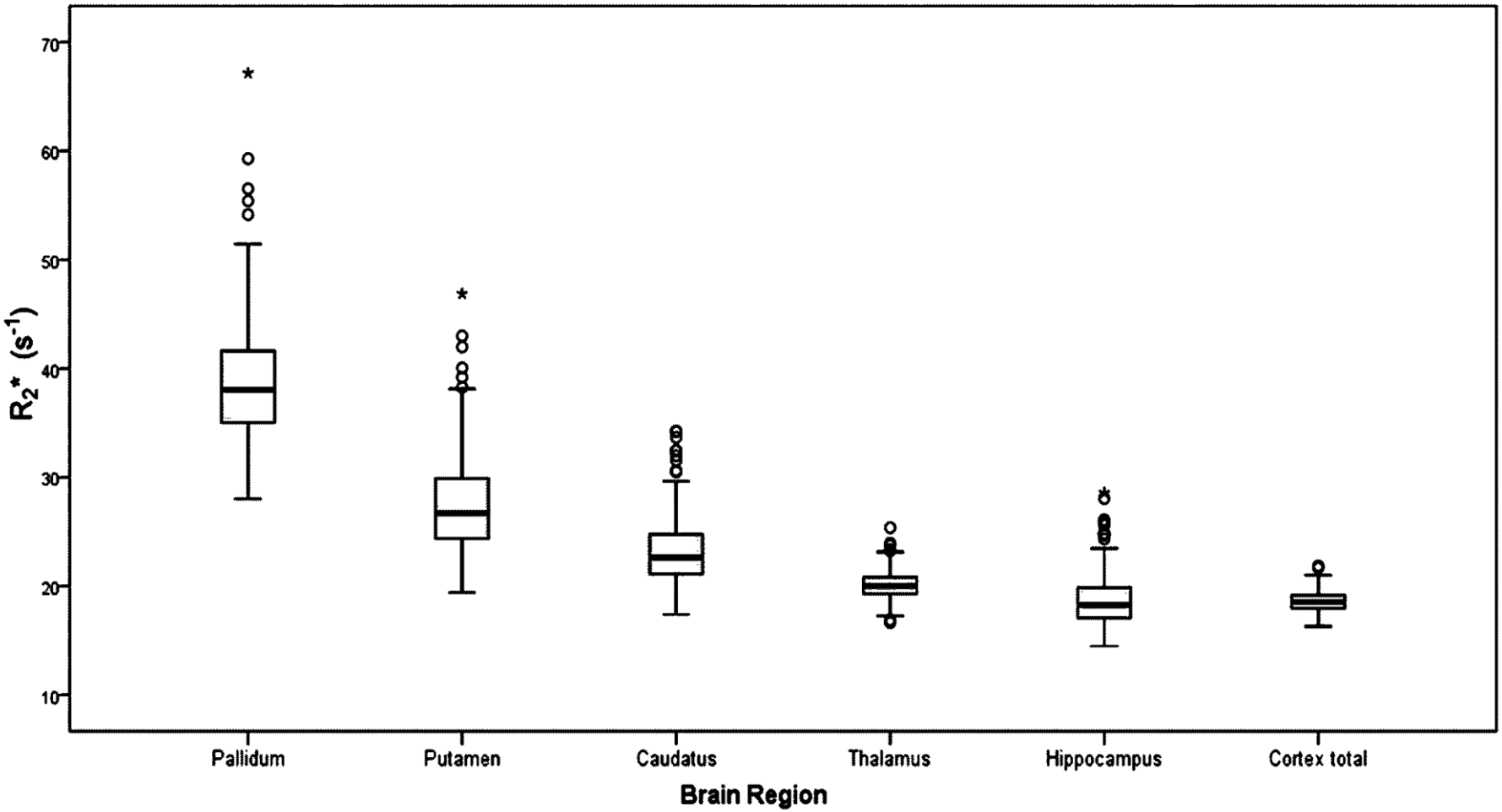

Figure 15. R2*-based iron in different brain regions. The median of regional R2* values is displayed by the central line within the box. Box edges represent the 25th and 75th percentile values. Whiskers extend to the most extreme data points not considering outliers. Outliers are displayed by circles and most extreme outliers by asterisks.

Table 4 presents the relationship between regional R2* values and cognition. As can be seen from this table, higher iron load in the pallidum related inversely with all cognitive measures, except memory. Furthermore, these associations remained significant after adjustment for multiple comparisons.

Other significant associations existed between R2* in putamen and global cognitive function and psychomotor speed. A further significant relationship existed between caudate nucleus and psychomotor speed, remaining not significant after adjustment for multiple testing. There were no significant relationships between R2* values in neocortex and hippocampus and cognition.

Figure 16 and **17** are scatterplots displaying the inverse relationship between R2*-based iron in the pallidum and in the putamen and domain-specific neuropsychological test scores.

Neither verbal nor figural memory as determined by “Bäumler´s Lern und Gedächtnis-Test” were significantly related to R2* values in the various brain regions including the hippocampus (data not shown).

When assessing the associations between regional iron content and cognitive functioning within tertiles of age, the associations were strongest in the highest age group ranging from 71 to 86 years **(Table 5)**. There existed an almost linear relationship between quartiles of R2* iron load in the pallidum and impairment in global cognitive function, executive function and psychomotor speed **(Figure 18)**. Similar results were seen between quartiles of R2* iron load in the putamen and psychomotor speed **(Figure 19)**.

None of the structural MRI changes mediated the relationship between R2* measured iron content in pallidum and putamen and cognition **(Table 6)**.

As determined by the method of Lindenberger and Pötter (Lindenberger, Pötter 1998) the R2* based iron content in the pallidum accounted for 9% of the age-related variance in executive function, 7% in global cognitive function and 8% in psychomotor speed results of study participants. R2* based iron load in the putamen accounted for 24% of the age-related variance in executive function, 18% in global cognitive function and 21% in psychomotor speed results.

	Global cognitive function (G-factor)				Memory			
Brain Region	**β**	**95% CI**	**p†**	**corrected p***	**β**	**95% CI**	**p†**	**corrected p***
Cortex	0,0193	[-0.037, 0.075]	0,50000	0,66182	0,0713	[-0.020, 0.163]	0,13000	0,331
Hippocampus	0,0046	[-0.015, 0.025]	0,65000	0,71373	0,0095	[-0.023, 0.042]	0,56000	0,665
Pallidum	-0,0192	[-0.029, -0.010]	**0,00008**	**0,00218**	-0,0134	[-0.029, 0.002]	0,09800	0,289
Putamen	-0,0165	[-0.029, -0.004]	**0,01000**	**0,04667**	-0,0075	[-0.028, 0.013]	0,48000	0,662
Caudatus	-0,0156	[-0.033, 0.002]	0,07900	0,26024	-0,0085	[-0.037, 0.020]	0,56000	0,665
Thalamus	0,0129	[-0.025, 0.050]	0,50000	0,66182	0,0352	[-0.026, 0.096]	0,26000	0,502

	Executive function				Psychomotor speed			
Brain Region	**β**	**95% CI**	**p†**	**corrected p***	**β**	**95% CI**	**p†**	**corrected p***
Cortex	0,0588	[-0.008, 0.126]	0,08600	0,26756	-0,0657	[-0.149, 0.018]	0,12000	0,32000
Hippocampus	0,0193	[-0.004, 0.043]	0,11000	0,30800	-0,0099	[-0.040, 0.020]	0,52000	0,66182
Pallidum	-0,0158	[-0.028, -0.004]	**0,00710**	**0,04667**	-0,0307	[-0.045, -0.017]	**0,00002**	**0,00118**
Putamen	-0,0189	[-0.038, -0.004]	**0,01300**	0,05600	-0,0275	[-0.046, -0.009]	**0,00330**	**0,03080**
Caudatus	-0,0145	[-0.035, 0.006]	0,17000	0,38080	-0,0275	[-0.053, -0.002]	**0,03400**	0,11900
Thalamus	0,0204	[-0.024, 0.065]	0,37000	0,57556	-0,0282	[-0.084, 0.028]	0,32000	0,54303

Table 4. Multivariate linear regression analysis[1]: R2* -based iron in different brain regions and cognitive functioning. [1]Adjusted for age, sex, education, hypertension, diabetes, cardiac diseases and family structure; † uncorrected p-value; *corrected p-value (Benjamini Hochberg FDR)

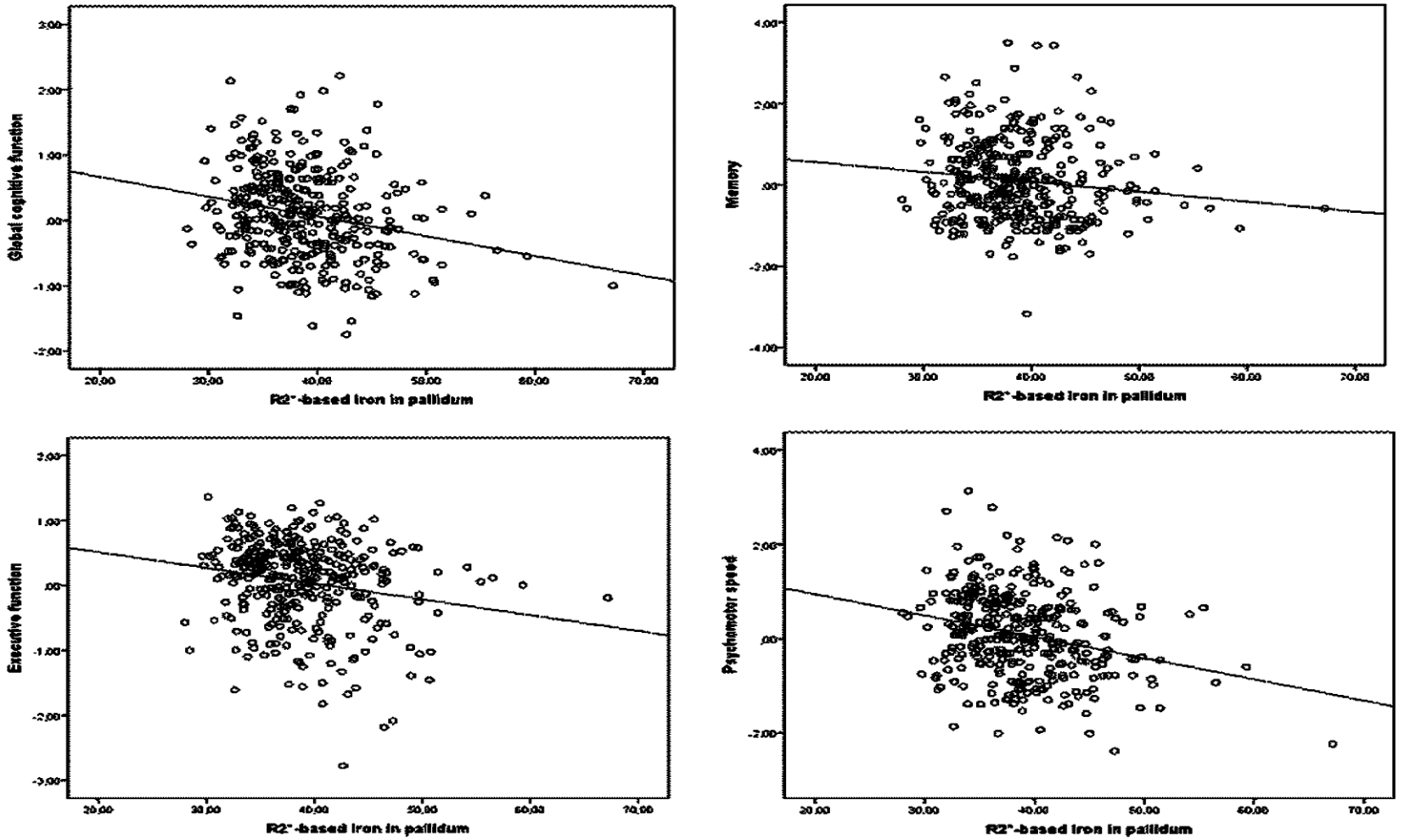

Figure 16. Scatterplots showing the correlation between R2*-based iron in pallidum and domain-specific neuropsychological test scores. X-axis displays z-values of neuropsychological test scores, y-axis gives R2*-based iron in pallidum. Pearson´s correlation showed significant associations of R2*-based iron with scores of global cognitive function (r=-0.231, p<0.001), memory (r=-0.129, p=0.019), executive function (r=-0.196, p<0.001), and psychomotor speed (r=-0.261, p<0.001).

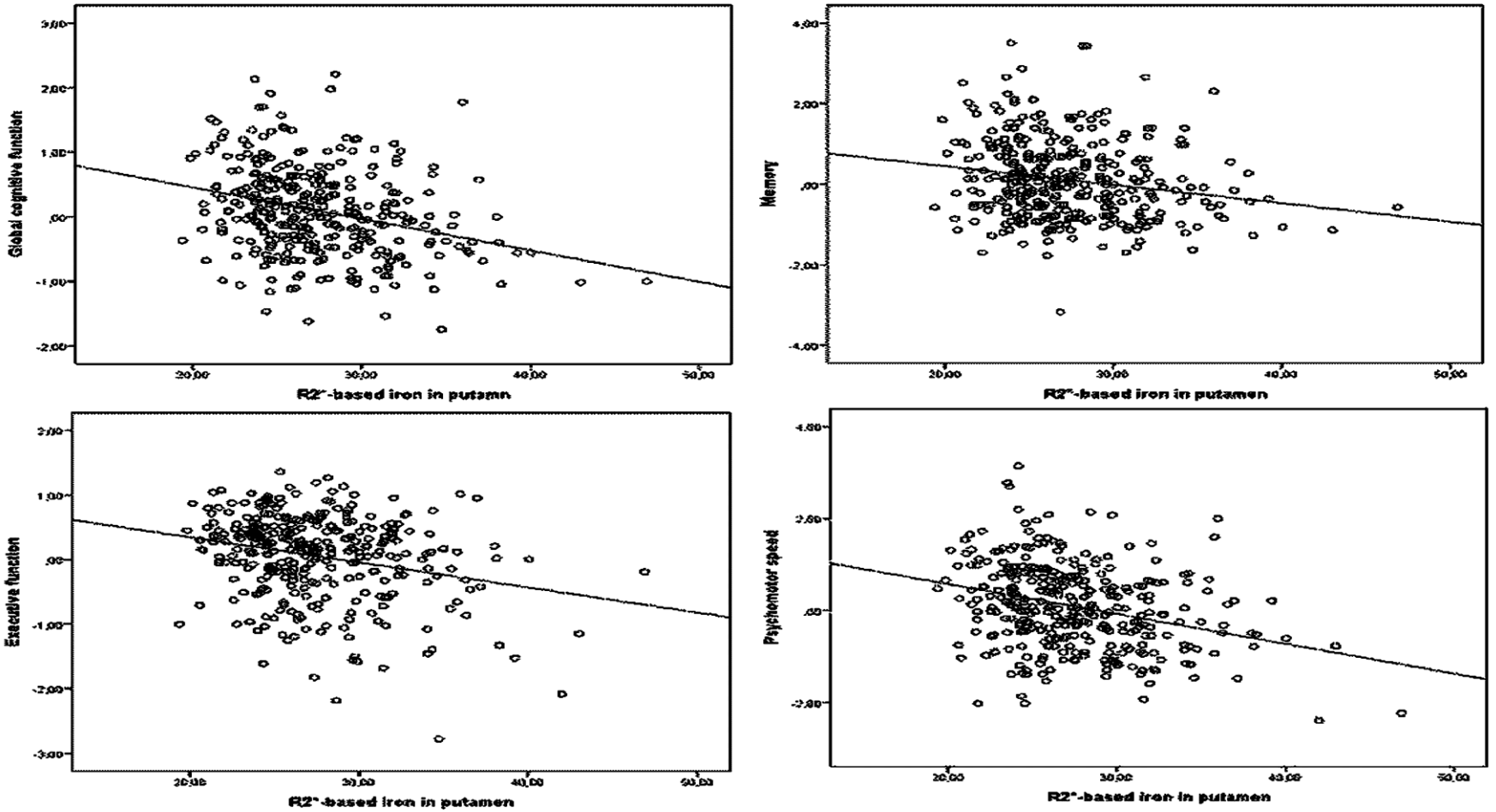

Figure 17. Scatterplots showing the correlation between R2*-based iron in putamen and domain-specific neuropsychological test scores. X-axis displays z-values of neuropsychological test scores, y-axis gives R2*-based iron in putamen. Pearson´s correlation showed significant associations of R2*-based iron with scores of global cognitive function (r=-0.295, p<0.001), memory (r=-0.192, p<0.001), executive function (r=-0.252, p<0.001), and psychomotor speed (r=-0.302, p<0.001).

Age Category 1 (38-60 years)

	Global cognitive function				Memory				Executive function				Psychomotor speed			
	beta	**CI**	**p**	**P corr.**	**beta**	**CI**	**p**	**P corr.**	**beta**	**CI**	**p**	**P corr.**	**beta**	**CI**	**p**	**P corr.**
Cortex	0.0166	[-0.07;0.10]	0.71	1	0.0001	[-0.16,0.16]	1	1	0.0576	[-0.02;0.14]	0.16	1	-0.0300	[-0.16;0.10]	0.66	1
Hippoc.	-0.0004	[-0.05;0.04]	0.99	1	0.0477	[-0.04;0.13]	0.26	1	-0.0136	[-0.06;0.03]	0.54	1	-0.0381	[-0.11;0.03]	0.28	1
Pallidum	-0.0021	[-0.02;0.02]	0.85	1	0.0014	[-0.04;0.04]	0.95	1	0.0009	[-0.02;0.02]	0.94	1	-0.0076	[-0.04;0.03]	0.66	1
Putamen	-0.0016	[-0.03;0.03]	0.91	1	-0.0005	[-0.05;0.05]	0.98	1	-0.0053	[-0.03;0.02]	0.7	1	0.0027	[-0.04;0.05]	0.9	1
Caudatus	0.0142	[-0.03;0.06]	0.51	1	0.0112	[-0.07;0.09]	0.78	1	0.0201	[-0.02;0.06]	0.31	1	0.0115	[-0.05;0.07]	0.72	1
Thalamus	0.0824	[-0.00;0.16]	0.05	0.6	0.0753	[-0.08;0.23]	0.34	1	0.1148	[0.04;0.19]	*0.004*	0.08	0.0524	[-0.08;0.18]	0.43	1

Age Category 2 (61-70 years)

	Global cognitive function				Memory				Executive function				Psychomotor speed			
	beta	**CI**	**p**	**P corr.**	**beta**	**CI**	**p**	**P corr.**	**beta**	**CI**	**p**	**P corr.**	**beta**	**CI**	**p**	**P corr.**
Cortex	-0.0193	[-0.11;0.08]	0.67	0.7	0.0610	[-0.09;0.21]	0.42	0.6	0.0343	[-0.08;0.14]	0.54	0.66	-0.1346	[-0.27;0.00]	0.056	0.247
Hippoc.	0.0204	[-0.02;0.06]	0.27	0.5	0.0374	[-0.02;0.09]	0.20	0.4	0.0295	[-0.01;0.07]	0.17	0.41	0.0144	[-0.04;0.07]	0.630	0.6997
Pallidum	-0.0154	[-0.03;-0.0]	*0.048*	0.2	-0.0025	[-0.03;0.02]	0.84	0.8	-0.0113	[-0.03;0.01]	0.22	0.44	-0.0302	[-0.05;-0.01]	*0.012*	0.2
Putamen	-0.0191	[-0.04;0.00]	0.065	0.2	0.0073	[0.02;0.04]	0.66	0.7	-0.0271	[-0.05;-0.0]	*0.025*	0.2	-0.0292	[-0.06;0.00]	0.072	0.247
Caudatus	-0.0092	[-0.04;0.02]	0.49	0.7	0.0171	[-0.02;0.06]	0.41	0.6	-0.0215	[-0.05;0.01]	0.16	0.41	-0.0117	[-0.05;0.03]	0.550	0.66
Thalamus	-0.0243	[-0.09;0.04]	0.46	0.6	0.0832	[-0.02;0.18]	0.11	0.3	-0.0296	[-0.10;0.04]	0.44	0.65	-0.1169	[-0.21;-0.02]	*0.019*	0.2

Age Category 3 (71-86 years)

	Global cognitive function				Memory				Executive function				Psychomotor speed			
	beta	**CI**	**p**	**P corr.**	**beta**	**CI**	**p**	**P corr.**	**beta**	**CI**	**p**	**P corr.**	**beta**	**CI**	**p**	**P corr.**
Cortex	0.0612	[0.04;0.17]	0.26	0.39	0.1588	[-0.00;0.32]	0.052	0.139	0.1113	[-0.04;0.26]	0.16	0.274	-0.0677	[-0.22;0.08]	0.39	0.551
Hippoc.	-0.0043	[-0.03;0.02]	0.76	0.793	-0.0140	[-0.06;0.03]	0.510	0.612	0.0237	[-0.01;0.06]	0.23	0.368	-0.0142	[-0.05;0.02]	0.48	0.6061
Pallidum	-0.0238	[-0.04;-0.01]	*0.0005*	**0.011**	-0.0193	[-0.04;0.00]	0.081	0.177	-0.0267	[-0.05;-0.01]	*0.009*	**0.037**	-0.0316	[-0.05;-0.01]	*0.001*	**0.016**
Putamen	-0.0209	[-0.04;-0.00]	*0.018*	0.054	-0.0249	[-0.05;0.00]	0.073	0.175	-0.0202	[-0.05;0.00]	0.11	0.22	-0.0319	[-0.05;0.01]	*0.009*	**0.037**
Caudatus	-0.0366	[-0.06;-0.01]	*0.005*	**0.037**	-0.0486	[-0.09;-0.01]	*0.018*	0.054	-0.0299	[-0.07;0.00]	0.12	0.221	-0.0487	[-0.08;-0.01]	*0.009*	**0.037**
Thalamus	-0.0155	[-0.07;0.04]	0.57	0.622	-0.0249	[-0.11;0.06]	0.560	0.622	0.0023	[-0.08;0.08]	0.95	0.95	-0.0320	[-0.11; 0.05]	0.42	0.56

Table 5. Analysis of the effects of iron accumulation on cognitive functions stratified by age.

Corrected for age, sex, education, hypertension, diabetes, cardiac diseases and family structure.
P corr.: corrected p-value (Benjamini-Hochberg FDR)
Uncorrected p-value < 0.05
Corrected p-value < 0.05

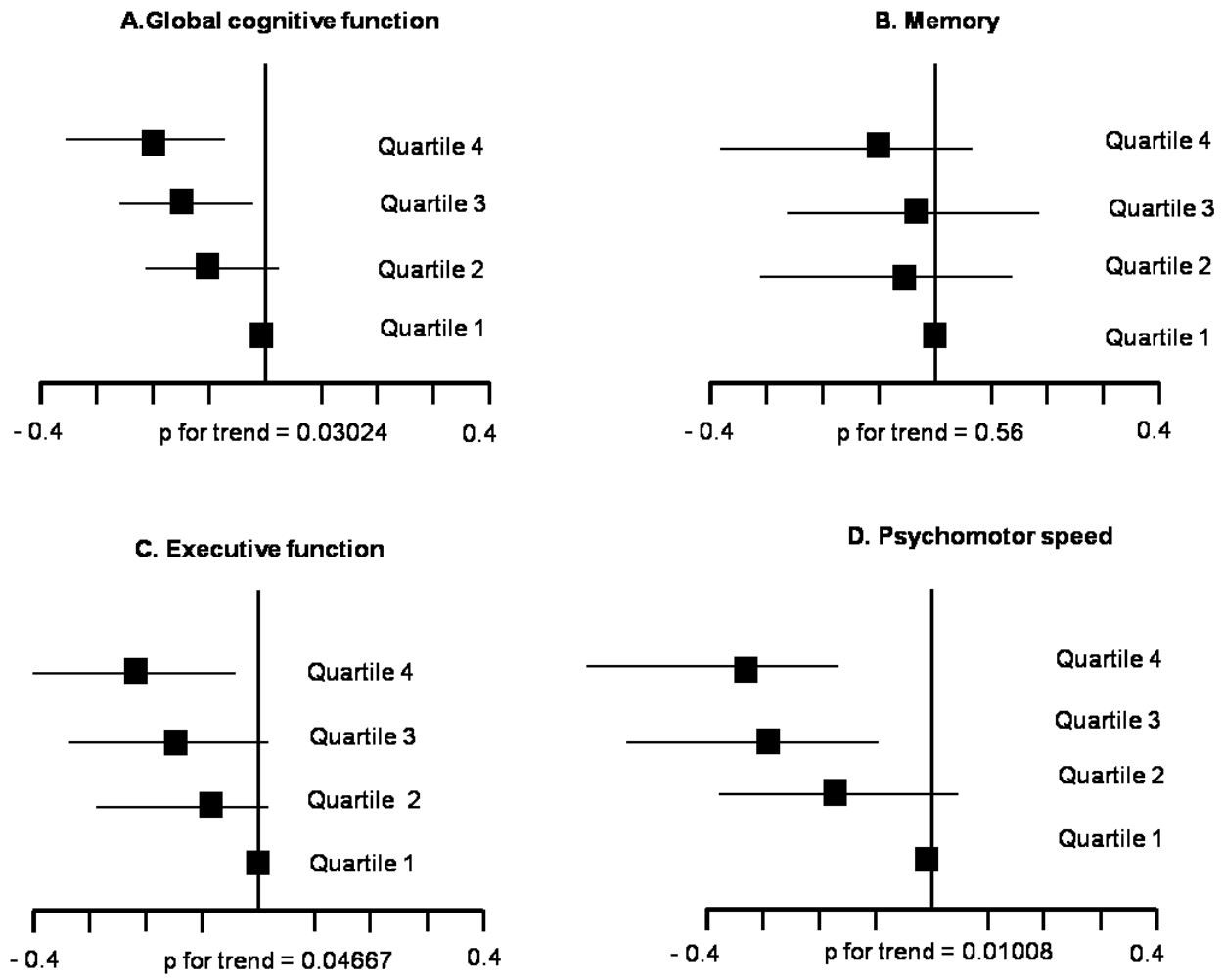

Figure 18. Iron load-related effects between R2*-based iron in pallidum and domain-specific cognitive performance. Mixed model analysis adjusted for age, sex, education, hypertension, diabetes, cardiac diseases and family structure displays the association between R2* quartiles in the pallidum on performance in all cognitive domains including global cognitive function, executive function, psychomotor speed and memory with the lowest quartile of the R2*-based iron distribution serving as the reference. The range of R2*-based iron values in quartiles 1, 2, 3, and 4 was 28.02 - 35.01, 35.02 - 38.09, 38.10 - 41.65 and 41.76 - 67.16 respectively. Squares on the x-axis indicate the β-coefficients and bars give the 95% confidence intervals. This figure demonstrates that increasing R2*-based iron in pallidum is related to worse performance in global cognitive function, executive function and psychomotor speed. An almost linear decline in these three cognitive categories occurred with increasing R2* quartile. The association for memory was non-significant.

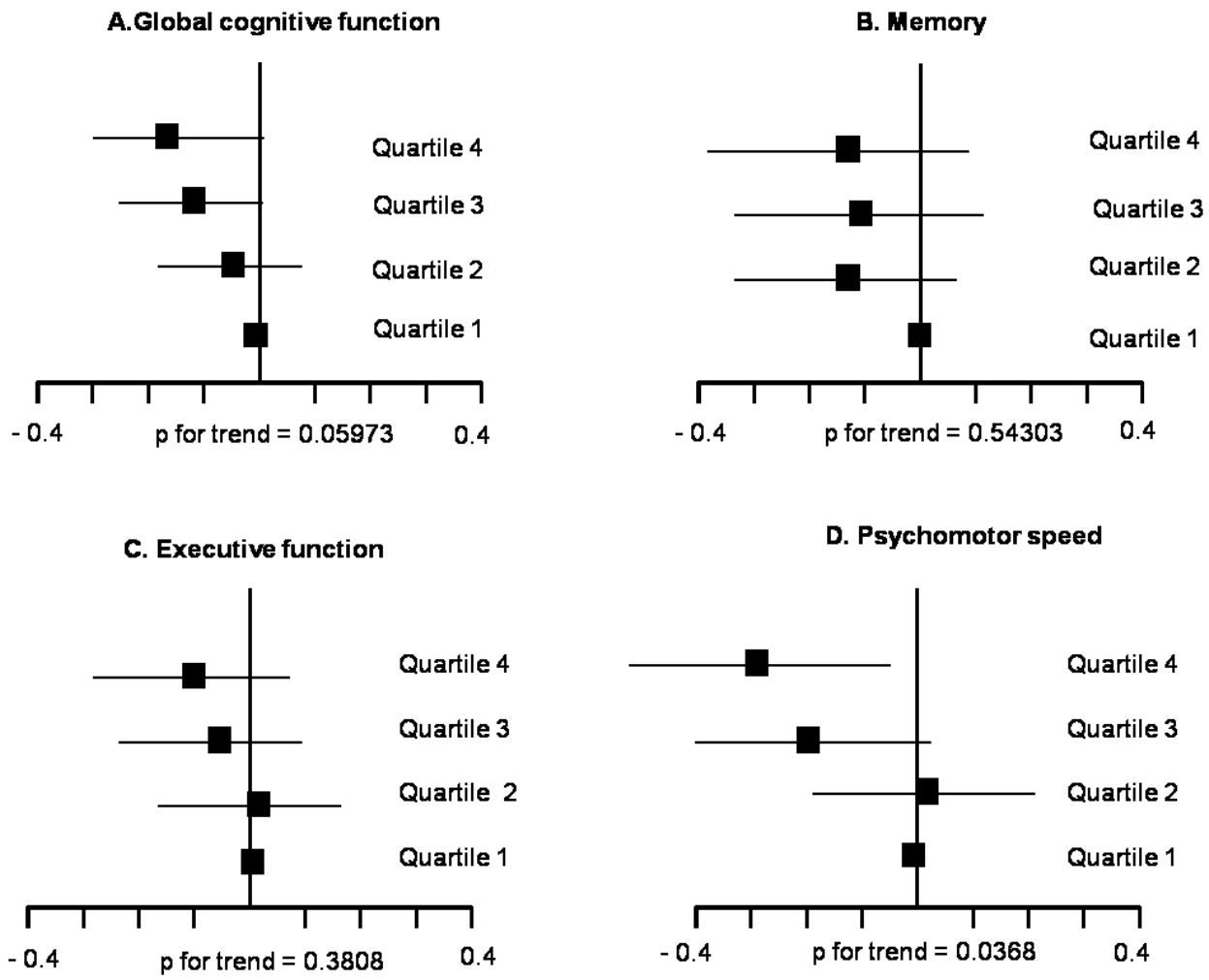

Figure 19. Iron load-related effects between R2*-based iron in putamen and domain-specific cognitive performance. Mixed model analysis adjusted for age, sex, education, hypertension, diabetes, cardiac diseases and family structure displays the association between R2* quartiles in the putamen on performance in all cognitive domains including global cognitive function, executive function, psychomotor speed and memory with the lowest quartile of the R2*-based iron distribution serving as the reference. The range of R2*-based iron values in quartiles 1, 2, 3, and 4 was 19.41 - 24.44, 24.45 - 26.74, 26.75 - 29.80 and 29.81 - 46.88 respectively. Squares on the x-axis indicate the β-coefficients and bars give the 95% confidence intervals. This figure demonstrates that increasing R2*-based iron in the putamen is related to worse performance in psychomotor speed. An almost linear decline in this cognitive category occurred with increasing R2* quartile. The association for global cognitive function, memory and executive function was non-significant.

R2* in Pallidum	Brain volume			
	Total effect	Direct effect	Indirect effect	Bootstrapped CI
Global cognitive function (n=328)	-0.0183	-0.0178	-0.0005	-0.0034; 0.0005
Executive function (n=333)	-0.0147	-0.0139	-0.0008	-0.0034; 0.0008
Psychomotor speed (n=333)	-0.031	-0.0304	-0.0006	-0.0045; 0.0006
	WMH volume			
Global cognitive function(n=297	-0.0183	-0.0177	-0.0025	-0.0028; 0.0004
Executive function(n=301)	-0.0157	-0.0159	0.0004	-0.0012; 0.002
Psychomotor speed (n=302)	-0.0326	-0.0316	-0.0044	-0.0044; 0.0009
	Number of lacunes			
Global cognitive function(n=330)	-0.0182	-0.0185	0.0003	-0.0001; 0.0013
Executive function(n=335)	-0.0147	-0.0147	0	-0.0007; 0.0002
Psychomotor speed (n=335)	-0.0305	-0.031	0.0005	-0.0001; 0.0017

R2* in Putamen	Brain volume			
	Total effect	Direct effect	Indirect effect	Bootstrapped CI
Global cognitive function (n=328)	-0.0168	-0.0159	-0.0009	-0.0041; 0.0004
Executive function (n=333)	-0.0188	-0.0175	-0.0013	-0.0052; 0.0008
Psychomotor speed (n=333)	-0.0271	-0.026	-0.0011	-0.0054; 0.0006
	WMH volume			
Global cognitive function(n=297	-0.0173	-0.0165	-0.0008	-0.0037; 0.0002
Executive function(n=301)	-0.0206	-0.0208	0.0002	-0.0005; 0.0022
Psychomotor speed (n=302)	-0.0265	-0.0253	-0.0012	-0.0058; 0.0012
	Number of lacunes			
Global cognitive function(n=330)	-0.017	-0.0173	0.0003	-0.0003; 0.0022
Executive function(n=335)	-0.0186	-0.0186	0	-0.0011; 0.0003
Psychomotor speed (n=335)	-0.0277	-0.0283	0.0006	-0.0005; 0.0032

Table 6. Mediation models[1] assessing the effect of brain volume, WMH volume and number of lacunes on the relationship between R2*-based iron in pallidum and putamen and cognitive performance.

Abbreviation: WMH volume= White matter hyperintensities volume

[1]Dependent variable: global cognitive function, executive function and psychomotor speed. Independent Variable: R2* in pallidum and in putamen; Mediators are brain volume, WMH volume and number of lacunes. The models are adjusted for age, sex, education, hypertension, diabetes and cardiac diseases.

Note zero was always within the bootstrapped CI of the indirect effect, indicating that brain abnormalities had no mediating effects on the association between pallidal and putaminal R2* and cognitive functioning. Due to missing data the effective sample size varied between 297 and 335.

4. DISCUSSION

In the present study, the largest investigation of brain iron accumulation in community-dwelling individuals performed so far, we observed, as previously reported, that the highest concentration of iron is in the pallidum, followed by putamen and caudate nucleus. These findings are in line with previous studies revealing similar results (Hallgren, Sourander 1958, Aoki, Okada et al. 1989, Drayer, Burger et al. 1986, Pfefferbaum, Adalsteinsson et al. 2009).

Generally, as earlier discussed in the introduction, brain iron accumulation starts at birth, when hardly any iron can be measured (Schenck, Zimmerman 2004, Taylor, Morgan 1990, Zecca, Gallorini et al. 2001) and increases rapidly during the first two decades of life in brain regions including globus pallidus, dentate, substantia nigra (pars reticulate), and red nucleus (Brass, Chen et al. 2006), but remaining relatively constant from 30 years onward and reaching maximum levels in old age (Bartzokis, Beckson et al. 1997). Interestingly, the thalamus accumulates iron to a minor extent (Brass, Chen et al. 2006), even showing a slight reduction in later life (Bartzokis, Beckson et al. 1997, Hallgren, Sourander 1958), an observation which we were able to confirm by our own results by showing a negative correlation between R2*-based iron in thalamus and higher age. Further findings revealed that increased iron content correlates with higher age in putamen, which has also been observed in an earlier study by Pfefferbaum and colleagues (Pfefferbaum, Adalsteinsson et al. 2010) showing the greatest evidence for higher iron deposition in advanced age for the putamen.

In contrast to the Bartzokis group (Bartzokis, Tishler et al. 2007), which found significant gender differences in brain iron values, we could not show any significant associations between brain iron content and sex. Bartzokis and colleagues (Bartzokis, Tishler et al. 2007) observed increased brain iron levels in men compared to women, suggesting that peripheral iron levels can probably influence brain iron amounts and may thus be a modifiable risk factor for age-related degenerative diseases. The possible relationship between peripheral iron levels and brain iron load was first noted by Hallgren and Sourander based on anecdotal observations made during their landmark post mortem study of human non-heme brain iron (Hallgren, Sourander 1958). The investigators noted that subjects with known hemorrhages or

severe anemia ante mortem had lower brain iron levels at post mortem and proposed that brain iron may be mobilized for metabolic needs outside the brain (Hallgren, Sourander 1958). It is generally known that levels of iron and ferritin differ between men and women, most likely resulting from female-specific effects such as menstruation, childbirth or genetic variation (Whitfield, Treloar et al. 2003, Milman 1996). Further, men tend to have an earlier age of unset and higher prevalence in age-related neurodegenerative disorders (Bartzokis, Tishler et al. 2004) such as PD (Van Den Eeden, Tanner et al. 2003, Haaxma, Bloem et al. 2007), DLB (Barker, Luis et al. 2002) and AD (Miech, Breitner et al. 2002, Raber, Huang et al. 2004, Alizadeh, Njajou et al. 2009). Moreover, men are also more prone to develop extrapyramidal (motor) side effects when treated with dopaminergic agents, proposing an increased susceptibility of their basal ganglia to toxicity (Bartzokis, Cummings et al. 1999). Bartzokis and colleagues (Bartzokis, Tishler et al. 2007, Bartzokis, Tishler et al. 2004) have suggested that increased brain iron levels in men may be to a certain extent responsible for the earlier age of neurodegenerative disease onset, probably mediated by prevalent iron metabolism genes (Bartzokis, Lu et al. 2010). In addition, a recent study (Tishler, Raven et al. 2012) examined the association between women who underwent hysterectomy, who are therefore predisposed to peripheral iron accumulation not occurring in other women, and brain ferritin iron. Hysterectomy was significantly associated with elevated iron load in the frontal lobe white matter, and iron levels in caudate and thalamus were also increased in women who had hysterectomies when compared with male control subjects. Nevertheless, it is important not to draw hasty conclusions from these findings, especially when regarding conditions of iron overload such as in hereditary hemochromatosis (HH) and related brain iron accumulation. Mutations in the HFE gene (Feder, Gnirke et al. 1996) are commonly associated with HH, which is an autosomal recessive disorder characterized by an excessive absorption of dietary iron resulting in abnormal iron accumulation, with secondary tissue damage in several organs such as liver, pancreas, and heart (Nandar, Connor 2011). Historically, the brain was assumed to be unaffected by the peripheral iron accumulation seen in hereditary hemochromatosis (Ke, Qian 2007). However, as discussed in the previous sections of the introduction, the presence of HFE gene variants was associated with higher risk of developing neurodegenerative diseases such as AD (Lee, Connor 2005,

Connor, Lee 2006, Moalem, Percy et al. 2000, Sampietro, Caputo et al. 2001, Pulliam, Jennings et al. 2003, Combarros, Garcia-Roman et al. 2003), PD and parkinsonism (Nielsen, Jensen et al. 1995, Buchanan, Silburn et al. 2002, Costello, Walsh et al. 2004, Papanikolaou, Samuels et al. 2004, Thomas, Jankovic 2004, Dekker, Giesbergen et al. 2003), as well as ALS (Wang, Lee et al. 2004, Mitchell, Freeman et al. 2009). Additionally, a further study (Bartzokis, Lu et al. 2010) revealed that the presence of HFE and/or transferrin gene variants was related to elevated brain ferritin iron in older men compared with non-carriers. Nonetheless, there exist also data contrary to the view that HFE genes mutations result simultaneously in brain iron accumulation and that HFE variants are a risk factor for neurodegenerative disorders. In a mouse model of hemochromatosis (Santos, Schilham et al. 1996), an abnormal iron accumulation was observed in the liver, whereas the level of brain iron was virtually normal (Moos, Trinder et al. 2000). Further support derives from another mouse model study for human hereditary hemochromatosis revealing no detection of brain iron accumulation by histopathology (Golub, Germann et al. 2005). In addition, a previous publication revealed that variations in brain transcripts related to Alzheimer's disease in a model of HFE hemochromatosis are not consistent with increased AD risk and the observed effects on AD-related gene transcripts might even be predicted to protect against AD to a certain degree (Johnstone, Graham et al. 2012). Moreover, also another publication questioned the role of HFE mutations in movement disorders, suggesting that movement disorders are rare in association with hereditary hemochromatosis (Russo, Edwards et al. 2004). At the moment, it is not totally clarified to which extend HFE gene mutations contribute to the development of neurodegenerative disorders and actually lead to brain iron accumulation caused by increased peripheral iron levels (Ke, Qian 2007). Therefore, our results do not support Bartzokis´ et al. suggestion that variations in brain iron levels due to differences in peripheral iron levels are caused by gender differences (Bartzokis, Tishler et al. 2007). Further support derives from a recent longitudinal study by Penke and colleagues (Penke, Valdes Hernandez et al. 2012). The authors of this study investigated the association between brain iron deposits and general cognitive ability in a healthy cohort, revealing that men showed a higher incidence and degree of iron deposits than women, but gender difference was not statistically significant ($p > 0.27$). In addition, all associations of iron deposits with cognitive

variables did not vary significantly between the genders (Penke, Valdes Hernandez et al. 2012). Additional data, in line with our results, derive from an earlier study (Xu, Wang et al. 2008), also observing no gender-related differences in brain iron levels in 78 healthy adults using magnetic susceptibility-weighted phase imaging.

The main results from our study demonstrate that iron load in specific brain regions is actually related to cognitive functioning. This was particularly true for iron in the pallidum and putamen, while associations between iron load in other brain regions and cognition were inconsistent. R2* based iron content in the pallidum accounted for 7% to 9% of the age-related variance in domain specific cognitive functions. The effect of putaminal iron load was considerably larger, it accounted for 18% of the age-related variance in global cognitive function and 24% in executive function. The association between iron in structures of the basal ganglia and cognition was strongest in the highest tertile of age ranging from 71 to 86 years. Although, the relationship between brain iron and cognition in non-demented individuals have mainly been neglected in the past, a small number of authors have investigated these associations over the last years, revealing comparable findings (Pujol, Junque et al. 1992, Sullivan, Adalsteinsson et al. 2009, Bartzokis, Lu et al. 2011, Rodrigue, Daugherty et al. 2013). As mentioned before, Penke and colleagues (Penke, Valdes Hernandez et al. 2012) investigated the association between brain iron deposits and general cognitive ability in a healthy cohort, suggesting an inverse relationship between iron deposits in the basal ganglia and cognition in the general population (Penke, Valdes Hernandez et al. 2012). The authors applied a novel, semi-automated analysis of MRI scans based on multispectral image fusion to quantify iron deposits in basal ganglia and microbleeds in 143 non-demented individuals, who were tested for general cognitive performance in childhood and in older age. The findings of this study suggest that iron deposits are not only a biomarker of general cognitive ability in old age and age-related cognitive decline, but that they are also associated with lifelong-stable trait of intelligence (Penke, Valdes Hernandez et al. 2012). Further, a latest publication by Li and colleagues explored the correlation between magnetic susceptibility, a surrogate marker of brain iron, of gray matter structures with behavioral measures of motor and cognitive ability in 132 healthy middle aged and older adults. The authors of this study revealed that independent of gender, age, and global cognitive function, increasing magnetic susceptibility in the

globus pallidus and red nuclei was related to decreasing manual dexterity (Li, Langkammer et al. 2014).

The mechanisms underlying the association between basal ganglial iron deposition and cognitive abilities are not clear. Further, it is not clarified to what extent iron accumulation contributes to the pathophysiology of age-related neurologic dysfunction and tissue damage observed in several disease states or whether iron represents only an epiphenomenon (Zecca, Youdim et al. 2004a). Our findings argue against the view that iron accumulation represents only an epiphenomenon of brain atrophy and vascular cerebral damage which by itself might be the cause for both, increased iron deposition and cognitive impairment. Our results further do not support the hypothesis made by the Penke group, suggesting that iron deposits could in fact be markers of small vessel dysfunction (Penke, Valdes Hernandez et al. 2012). In such instance iron load in the pallidum as well as in the putamen should have been related to these brain abnormalities and, moreover the association between pallidal and putaminal iron and cognition should have been mediated by either focal or diffuse brain abnormalities or both. We failed to observe such relationships and thus contrast a recent study in patients with CADASIL, in which cerebral small vessel disease-related brain lesions were associated to basal ganglionic iron accumulation (Liem, Lesnik Oberstein et al. 2012). A reason for these discrepant results may be that the amount of brain lesions in CADASIL patients by far exceeds the magnitude of cerebral damage that occurs with advancing age. The lack of an association between iron load, white matter damage and brain atrophy in our study is also against the view that age-related demyelination of white matter tracts or cortical pathology or atrophy may lead to increased iron release and finally contribute to chronic accumulation of iron in the basal ganglia (Bartzokis, Tishler et al. 2007, Schneider, Dusek et al. 2013).

A causal relationship between iron load and cognition cannot be inferred by our data. It is of note however, that increasing iron content in the basal ganglia related to increasing executive dysfunction and psychomotor speed dysfunction. No such association was seen for memory. This pattern of cognitive dysfunction is consistent with involvement of frontal-subcortical circuits (Bonelli, Cummings 2007, Duering, Zieren et al. 2011, Krause, Mahant et al. 2012) which originate in the prefrontal cortex, project to the striatum, connect to the pallidum and substantia nigra and from

there to the thalamus (Alexander, DeLong et al. 1986, Purves, Augustine et al. 2012). Moreover, we found an almost linear association between the amount of R2*-determined iron in pallidum as well as in putamen and cognitive performance, which also suggests a close relationship between the two measures.

Multiple mechanisms may be responsible for direct damaging effects of increased iron load. As already mentioned in the previous sections of the introduction, increasing amounts of low molecular weight, redox-active iron are potentially neurotoxic through the ability to induce oxidative reactions (Gutteridge 1994, Zecca, Youdim et al. 2004a, Richardson 2004, Honda, Casadesus et al. 2004) and by catalyzing the formation of ROS (Hagemeier, Geurts et al. 2012). The resulting oxidative stress can lead to the oxidation of cellular components causing the modification of DNA, proteins, lipids, and carbohydrates, and the subsequent oxidative damage is often connected with cell death either by necrosis or by apoptosis (Dalle-Donne, Giustarini et al. 2003). Iron- derived ROS may lead to membrane damage, abnormal cell signalling, mitochondrial and proteosomal dysfunction, electrophysiological imbalances and synaptolysis (Schipper 2004). Other devastating effects of increased brain iron levels include the aggregation of α-synuclein in neurons which is associated with several neurodegenerative diseases, including Parkinson disease (Kostka, Hogen et al. 2008). Moreover, there is evidence that metallochemical reactions resulting in formation of ROS might be the common denominator underlying a spectrum of neurodegenerative diseases including Alzheimer's disease, amyotrophic lateral sclerosis, prion disease, cataracts, mitochondrial disorders (Friedreich's ataxia), and Parkinson's disease (Bush 2000, Hirsch, Faucheux 1998, Jellinger 1999). Since iron is the most abundant transition metal in the brain, and biology in general, it is regarded as the most effective potential toxin (Thompson, Shoham et al. 2001).

In contrast to other studies that investigated brain iron accumulation on cognition (Pujol, Junque et al. 1992, Sullivan, Adalsteinsson et al. 2009, Bartzokis, Lu et al. 2011, Rodrigue, Daugherty et al. 2013, Li, Langkammer et al. 2014), we used a different technique to measure brain iron deposition. Moreover, unlike Penke and colleagues, who used the multispectral image fusion method and who were not able to distinguish brain microbleeds from iron deposits (Penke, Valdes Hernandez et al. 2012), we used mapping of the relaxation rate R2*, a well-known, easy applicable

technique, which has recently been confirmed in a post-mortem study (Langkammer, Krebs et al. 2010). In addition, according to a latest publication (Langkammer, Ropele et al. 2014) that compared available MR techniques for quantitative iron mapping in the brain, R2* -based imaging was revealed as the best validated technique for the detection of brain iron. Nevertheless, a limitation of this quantitative MRI technique includes the unspecific measurement of iron, in which primary ferritin which stores iron in a non–toxic but bioavailable form, is measured. However, at this point methods for specific measurement of iron in its toxic form are not available. On the other hand, it has been proposed that iron could be mobilized from ferritin by oxidative stress, following localized protein unfolding, or ferritin degradation within lysosomes. Moreover, ferritin itself can also experience degradation by the proteasome following either iron depletion or ferritin oxidation (Galaris, Pantopoulos 2008). In addition, in a previous study transgenic mouse lines were created, in which ferritin levels were selectively increased within dopaminergic (DA) neurons of the substantia nigra (SN), causing an increase in ferritin-bound iron within these cells (Kaur, Yantiri et al. 2003). In young animals, this process was considered to be protective against neurodegeneration induced by two common used toxin animal models of Parkinson's disease, systemic MPTP (Kaur, Yantiri et al. 2003) and paraquat administration (McCormack, Atienza et al. 2005), whereas in older animals prolonged ferritin elevation was found to lead to a selective age-related neurodegeneration of these cells and to worsen MPTP neurotoxicity (Kaur, Rajagopalan et al. 2006). Further, it has been noticed that iron regulatory protein 2-deficient (IRP2 −/−) mice suffer from age-related neurodegeneration as well, a finding probably attributable to elevated ferritin levels within degenerating neuronal populations in these animals (LaVaute, Smith et al. 2001). Smith and colleagues observed that IRP1+/−, IRP2−/− mice experienced an even more severe age-related neurodegeneration accompanied by still higher levels of ferritin accumulation (Smith, Cooperman et al. 2004). Thus it has been hypothesized that iron maintained primarily in a ferritin-bound state may lead to a labile iron pool (LIP) deficiency which could interfere on cellular function by reducing the amount of easily available iron required for the synthesis of important iron-sulfur containing enzymes including those of the mitochondria (Rouault 2001). Consequently, in order to evaluate whether LIP reduction is responsible for age related-neurodegeneration, Kaur and colleagues

observed LIP levels within DA SN neurons from ferritin-expressing transgenics versus wild-type mice with increasing age (Kaur, Rajagopalan et al. 2009). The observations of this study revealed that LIP levels were reduced in young ferritin transgenics versus age-matched controls, conversely, increased LIP levels were observed in older transgenic animals at the age at which selective neurodegeneration is first noted. The authors of this study further suggest that neurodegeneration is caused by increased rather than decreased LIP levels (Kaur, Rajagopalan et al. 2009). Several studies have demonstrated that ferric iron stored within the ferritin core can be easily reduced by cytotoxic byproducts of dopamine oxidation within dopaminergic neurons, allowing its release from ferritin as ferrous iron (Thomas, Aust 1986, Monteiro, Winterbourn 1989, Kienzl, Puchinger et al. 1995, Linert, Herlinger et al. 1996, Double, Maywald et al. 1998, Comporti 2002). In the case of the older ferritin transgenics, heavily iron-loaded ferritin localized within striatal axons might be degraded within lysosomes, releasing iron and increasing the LIP (Rouault 2001). A further contribution to the elevated labile iron pool seen in older ferritin transgenic mice could derive from the normal increase in age-related autophagy (Ward 2002) in the presence of increased ferritin-bound iron levels. An additional potential explanation of increased DA LIP include an increase in levels of H-ferritin-rich heteropolymers (or even H-ferritin homopolymers) that are less efficient at long-term chelation of iron. A combination of elevated H-ferritin levels coupled with age-related brain increases in iron, oxidative stress and autophagy could all contribute to dopaminergic labile iron pool increase and following neurodegeneration of this brain region (Kaur, Rajagopalan et al. 2009). We are aware that the results just mentioned, derive from animal or in vitro studies in which neurodegenerative conditions are induced or cytotoxic byproducts are applied, consequently creating a situation that differs from conditions of the normal aging brain. Anyway, more research is definitely needed to further clarify the exact mechanisms involved in these processes.

A further limitation of our investigation lies in its cross sectional design which does not permit to conclusively infer on a cause-effect relationship.

We realize that although statistically significant, the magnitude of cognitive impairment associated with increasing iron load seen in our study was small. The clinical importance of iron accumulation during normal aging is currently unknown.

Longitudinal studies are now needed to explore if increasing iron content in the basal ganglia indeed reflects age-related neurodegenerative processes which may ultimately result in clinically relevant cognitive impairment or even dementia. Data on the rate of iron accumulation during normal aging are yet barely available.

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