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Iron neurochemistry in Alzheimer's disease and Parkinson's disease: targets for therapeutics

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Abbreviations used: AD, Alzheimer´s disease; APP, Amyloid precursor protein; Aβ, Amyloid-beta; ApoE, Apolipoprotein E; PD, Parkinson´s disease; Substantia nigra, SN.

Abstract

Brain iron homeostasis is increasingly recognized as a potential target for the development of drug therapies for aging-related disorders. Dysregulation of iron metabolism associated with cellular damage and oxidative stress is reported as a common event in several neurodegenerative disorders such as Alzheimer´s, Parkinson´s and Huntington´s diseases. Indeed, many proteins initially characterized in those diseases such as amyloid-β protein, α-synuclein and huntingtin have been linked to iron neurochemistry. Iron plays a crucial role in maintaining normal physiological functions in the brain through its participation in many cellular functions such as mitochondrial respiration, myelin synthesis, and neurotransmitter synthesis and metabolism. However, excess iron is a potent source of oxidative damage through radical formation and due to the lack of a body-wide export system, a tight regulation of its uptake, transport and storage is crucial in fulfilling cellular functions while keeping its level below the toxicity threshold. In this review, we discuss the current knowledge on iron homeostasis in the brain and explore how alterations in brain iron metabolism affect neuronal function with emphasis on iron dysregulation in Alzheimer´s and Parkinson´s diseases. Finally, we discuss recent findings implicating iron as a diagnostic and therapeutic target for Alzheimer's and Parkinson's diseases.

Introduction

Iron plays a fundamental role in maintaining several biological functions in all living organisms, which include oxygen transport, mitochondrial respiration, cell growth and differentiation as well as the active site of metalloenzymes (Beard 2001). Within the brain, iron is crucial for maintaining the high metabolic and energetic requirements of neuronal tissues and is also involved in myelin synthesis, neurotransmitter synthesis and metabolism (Gerlach *et al.* 1994). The biological function of iron relies on its redox potential, which

allows the reversible transition from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state, thus catalyzing electron transfer reactions. However, the same redox chemistry can trigger deleterious reactions with oxygen and hydrogen peroxide leading to the formation of the highly reactive and damaging hydroxyl radicals via Haber-Weiss and Fenton chemistry. Therefore, iron is maintained in a bound and safe environment as a complex within metalloproteins that allows electron transfer reactions to occur and reduces its ability to produce reactive oxygen species (ROS), which can cause DNA and protein damage, lipid peroxidation and cellular death as observed in neurodegenerative diseases (Bush 2003, Barnham *et al.* 2004, Hare *et al.* 2013a).

It is well accepted that aging is the major risk factor for neurodegenerative disorders. Likewise, iron also accumulates in several brain regions in healthy aging and to a major extent in the brain of patients with several neurodegenerative disorders (Zecca *et al.* 2004b, Hare et al. 2013a, Ward *et al.* 2014). However, iron neurochemistry and its involvement in the pathology of neurodegenerative disorders has only received emphasis in the last two decades, partially due to disagreement in the literature as to whether iron increase is a primary or secondary event in neurodegenerative diseases. However, the recent progress in establishing diagnostic and treatment approaches for several neurodegenerative diseases has fostered a central role for iron in the process of neurodegeneration and associated oxidative stress. In this review, we discuss current knowledge on iron homeostasis in the brain and emphasize the importance of tight regulation of its uptake, transport and storage especially due to the lack of a body-wide excretion system. We further summarize the major events involved in the neurodegenerative disorders Alzheimer´s disease (AD) and Parkinson´s disease (PD) and debate their relationship to iron homeostasis. Finally, we discuss recent advances implicating a central role of the iron protein ferritin and iron chelation therapy in the diagnosis and treatment, respectively, of AD and PD.

Peripheral iron homeostasis

Total body iron in healthy adults normally ranges between 3.5 and 5.0 g, which is mostly stored within the liver (1800 mg) and red blood cells (RBC; 1800 mg) followed by macrophages (600 mg), bone marrow (300 mg) and muscles (300 mg) while very low amounts are found in kidney and brain (Winter *et al.* 2014). The only route of iron acquisition is through dietary intake and absorption of ferrous and heme iron by the intestinal enterocytes

(approximately 1-2 mg daily). Due to the lack of an active excretion system for iron in the body, most of the iron is recycled within the body from senescent RBCs within the spleen and bone marrow and the remainder is stored in ferritin/hemosiderin (Recalcati *et al.* 2012).

The iron cycling system within the periphery involves the actions of four different cells: intestinal enterocytes, erythroblasts, splenic macrophages and hepatocytes (Wang & Pantopoulos 2011, Winter et al. 2014). Iron uptake starts at the apical membrane of the intestinal enterocytes, which absorb ferrous iron through the successive reduction of ferric iron by duodenal cytochrome b (Dcytb) and cytosolic import via the divalent metal ion transporter-1 (DMT1) (Fig. 1). Enterocytes absorb also heme via the heme carrier protein-1 (HCP-1), with subsequent release of ferrous iron via the cytosolic heme oxygenase (HO-1). Cytosolic ferrous iron within the enterocytes is either oxidized by the H-chain of ferritin and stored in ferritin, or exported through ferroportin at the basal membrane site (De Domenico *et al.* 2011). Ferroportin-mediated iron export into extracellular fluids requires the oxidation of iron from the ferrous to the ferric form, which is carried out in enterocytes by the enzyme hephaestin, thus allowing the iron transport protein transferrin to bind two ferric ions and to deliver iron to the periphery (Fig. 1).

Transferrin is taken up by bone marrow erythroblasts, which express the transferrin receptor 1 (TfR1), which binds two transferrin proteins. When transferrin binds to TfR1, the complex is internalized with DMT1 receptors present within the membrane. The low pH of the endosome triggers release of ferric iron from transferrin, which is reduced to ferrous ions via Six-Transmembrane Epithelial Antigen of the Prostate-3 (STEAP-3) and released in the cytosol via DMT1 (Knutson 2007). The TfR1 is recycled back to the plasma membrane followed by apo-transferrin release from the cell. Cytosolic iron enters the mitochondrion where ferrous iron is used for heme synthesis, which is added to the globin chain in the cytosol to produce hemoglobin. The recovery of iron from hemoglobin involves recycling of damaged RBCs via the spleen macrophages (Recalcati et al. 2012). During phagocytosis, iron is released from heme via HO-1 and transferred from the phagolysosome via DMT1 to the cytosol. Similar to enterocytes, iron is stored in macrophages as ferric iron within ferritin or exported through ferroportin. In contrast to enterocytes, macrophages require the ferroxidase activity of the plasma protein ceruloplasmin (CP) instead of hephaestin for transferrin loading. Hepatocytes monitor and regulate iron availability in the periphery through production and secretion of hepcidin (Anderson *et al.* 2009). Hepatocytes are also able to assess transferrin saturation through TfR1 and transferrin receptor 2 (TfR2) in a homeostatic process modulated by the

hemochromatosis gene product (HFE) (Fig. 1). Under high levels of transferrin saturation, hepatocytes increase synthesis and release of hepcidin, which binds to ferroportin in iron-exporting cells and triggers ferroportin internalization and degradation, thus reducing iron absorption from enterocytes and iron release from macrophages. When iron saturation of transferrin is low, hepcidin secretion is reduced, allowing ferroportin to transport iron (Fig. 1). Mutations in HFE or TfR2 genes result in a lack of perception of transferrin saturation and increased iron absorption causing hemochromatosis type 1 and type 3 respectively (Nandar & Connor 2011). In addition to being controlled by hepatocyte iron levels and transferrin saturation, hepcidin secretion from hepatocytes is also regulated by three major factors including inflammation, erythropoietic activity and the oxygen tension within hepatocytes (Winter et al. 2014). At the transcriptional level, iron uptake and release is controlled by modulation of DMT1 and ferroportin mRNA levels via the transcription factor hypoxia-inducible factor-2A (HIF-2A) in response to changes in oxygen tension (Silvestri *et al.* 2008). Accordingly, DMT1 and ferroportin mRNA transcription are increased in response to decreased oxygen tension in enterocytes and hepatocytes (Winter et al. 2014).

In the extracellular milieu, high transferrin levels and its high affinity for iron ensure an almost complete sequestration of iron in a tight and non-labile form referred to as transferrin bound iron (TBI) leaving less than 1% of circulating iron not bound to transferrin and is referred to as non-transferrin bound iron (NTBI) (Breuer *et al.* 2000, Hare et al. 2013a, Cabantchik 2014). The NTBI is formed by coordinating several low molecular ligands such as ascorbate, phosphate, citrate and ATP, which due to their low affinity for iron in comparison to transferrin, constitute a labile form of iron, which can be targeted by chelation therapies in iron overload and iron toxicity disorders (Breuer *et al.* 2001, Brissot *et al.* 2012, Breuer *et al.* 2012). Extracellular body fluids function primarily as an iron-distributing system ferrying iron in a relatively safe and redox-inactive form and ensuring its delivery to the different cell compartments of the organism. In contrast, cellular iron homeostasis differs from the periphery and requires a more dynamic and accessible iron pool, which can fulfil its metabolic requirements (Breuer *et al.* 2008, Cabantchik 2014). Thus, the intracytoplasmic labile iron pool (LIP) plays a crucial role in cellular compartments as a metabolic source and, most importantly, as an indicator of the iron status of the cell.

At the translational level, cells sense changes in the LIP and respond by regulating the expression of proteins required for import, storage and export of iron according to their metabolic requirement (Cairo & Recalcati 2007, Cabantchik 2014). Cells utilise two iron

response proteins (IRP1/2) as cytosolic sensors of the LIP, which bind to iron regulatory elements (IREs) located in stem-loop structures within the untranslated mRNA (UTR) of iron-responsive proteins resulting either in mRNA stabilization and increase in translation by binding to the 3´-UTR of mRNA (TfR1/DMT1) or impair the translation by binding to IREs present at the 5´-UTR of mRNA (ferritin, ferroportin, α-synuclein, APP) (Anderson *et al.* 2012). Accordingly, this iron regulatory system increases IRP binding to IRE under conditions of low cellular iron and leads to increase in iron uptake through TfR1 and DMT1, while iron storage through ferritin and iron export through ferroportin are reduced. Conversely, when cellular iron levels are high, labile iron binds to IRPs and prevent their interaction with IREs, resulting in an increase in iron storage and export through translation of ferroportin and ferritin mRNA, while iron uptake is reduced through degradation of TfR1/DMT1 mRNAs (Muckenthaler *et al.* 2008, Hare et al. 2013a, Wong & Duce 2014).

Brain iron homeostasis

Overview

Iron plays a crucial role in maintaining normal physiological functions in the brain through its participation in many cellular functions such as mitochondrial respiration, myelin synthesis, and neurotransmitter synthesis and metabolism (Singh *et al.* 2014, Moos *et al.* 2007). Iron distribution within the brain is heterogeneous and the highest concentrations are found in the substantia nigra (SN) pars compacta and basal ganglia, which reach levels comparable to the liver (Drayer *et al.* 1986, Griffiths & Crossman 1993, Haacke *et al.* 2005, Singh et al. 2014). Aging in healthy individuals is accompanied by iron accumulation in several brain regions, with iron accumulation mainly bound to ferritin and neuromelanin (Connor *et al.* 1992b, Zecca *et al.* 2001, Zecca *et al.* 2004a). However, several neurodegenerative disorders such as AD (Connor *et al.* 1992a, Zhu *et al.* 2009, Smith *et al.* 2010, Qin *et al.* 2011), PD (Lhermitte *et al.* 1924, Dexter *et al.* 1987, Castellani *et al.* 2000, Ayton *et al.* 2014) and Huntington´s disease (Bartzokis *et al.* 2007, Cherny *et al.* 2012, Chen *et al.* 2013) are also characterized by increases in brain iron levels in specific regions, which is accompanied by cellular damage and oxidative stress (Zecca et al. 2004b, Barnham et al. 2004, Greenough *et al.* 2013). However, whether iron accumulation is a primary or a secondary event in aging-related neuronal death and the underlying mechanisms responsible for those changes are still not fully understood.

High expression levels of TfR1 on the luminal side of brain capillary endothelial cells (BCECs) and recent studies on iron trafficking within the brain support the concept that the first step in iron transport into the brain is mediated by transferrin uptake and endocytosis through the BCECs at the blood-brain barrier (BBB) (Fig. 2) (Crichton & Ward 1992, Moos et al. 2007). However, subsequent steps of iron release at the abluminal side of the BCECs into the brain interstitium are highly debated and two main hypotheses are proposed based on the involvement of DMT1 in this process: (1) the transcytosis model, which is based on the lack of DMT1 staining in BCECs (Moos & Morgan 2004, Moos et al. 2007), proposes that transferrin is transported into endosomes across the BCECs cytosol and is directly released into the brain (Fig. 2, red arrows). However, the transcytosis model is not supported by a quantitative transport of transferrin through the BBB (Crowe & Morgan 1992, Strahan *et al.* 1992, Moos *et al.* 2006). Moos et al. (2007) proposed that the tight association of astrocytes with BCECs may contribute to create a favoured microenvironment for iron release from transferrin at the abluminal side of BCECs and iron transport as low molecular weight complexes with citrate, ATP or ascorbate, which are secreted by astrocytes (Moos et al. 2007, Ward et al. 2014). (2) The receptor-mediated transferrin endocytosis model proposes that iron is released from endosomes in BCECs cytosol via DMT1 and exported into the brain interstitium through ferroportin (Fig. 2, blue arrows). This model provides also a mechanism accounting for the iron requirements of the BCECs and is supported by studies in the Belgrade rat, which demonstrated the presence of DMT1 in endothelial cells of the brain microvasculature (Burdo *et al.* 2001), as well as other studies that detected ferroportin and ferroxidases in cultured brain endothelial cells (Wu *et al.* 2004, McCarthy & Kosman 2013, McCarthy *et al.* 2014). A recent study has also supported the role of DMT1 in iron uptake at the BBB and suggested a new model for brain iron uptake in which the BCECs are not considered as a simple conduit for iron transport into the brain (Simpson *et al.* 2015). Accordingly, this model proposes BCECs as a site of regulation of brain iron homeostasis through regulation of transferrin uptake and recycling, and assigns an important role for astrocytes in iron uptake from BCECs and further redistribution within the brain (Simpson et al. 2015). Astrocytes do not express TfR1 but express ferroportin and glycophophatidylinositol-anchored CP, which oxidizes ferrous iron and facilitates binding to circulating transferrin (Wong *et al.* 2014a). Evidence for the major role of astrocytes in relaying iron to neurons is supported by the observation that CP inactivation in mice results in iron accumulation mostly in astrocytes and no iron accumulation in oligodendrocytes and large neurons of the deep nuclei is observed, while Purkinje neurons upregulate DMT1

indicating signs of iron deprivation probably because of iron sequestration in astrocytes (Patel *et al.* 2002). Transferrin is secreted in the brain by the choroid plexus, and neurons express TfR1, DMT1 and ferroportin, thus neurons are thought to acquire most of their iron from circulating transferrin and export excess iron through ferroportin, which is facilitated by circulating CP (Fig. 2) (Deane *et al.* 2004, Donovan *et al.* 2005, Rouault *et al.* 2009, Wong et al. 2014a).

Although transferrin is considered as the major iron transport and delivery protein in the periphery and it is the primary iron acquisition source for the brain, iron homeostasis in the brain is relatively independent of peripheral iron levels. Iron saturation of CSF transferrin is believed to be maintained close to saturation while that of serum is estimated at 30% and reaches saturation only in a disease like hemochromatosis, in which NTBI is detected in plasma (Batey *et al.* 1980, Brissot *et al.* 1985, Bradbury 1997, Moos et al. 2007). Thus the buffering capacity of transferrin in the brain is very limited and additional mechanisms for iron binding and storage are required in the brain to protect neurons from excess iron. Mouse models of both hypotransferrinemia, and of loss of function of ferroportin, showed minimal change in brain iron levels (Beard *et al.* 2005, Donovan et al. 2005) despite profound changes in peripheral iron levels. Thus, the brain is subject to privileged iron regulatory systems compared to peripheral organs.

Cellular distribution of brain iron

In normal adult brain, oligodendrocytes are the principal cells that stain for iron, which correlates also with high ferritin immunostaining, and they are the only cells to produce myelin (Connor *et al.* 1995b, Connor & Menzies 1996, Ortiz *et al.* 2004). Iron deficiency, which is the most common nutrient deficiency in the world, is associated with major neurological abnormalities, which are mostly attributed to hypomyelination (Todorich *et al.* 2008). Iron is an essential component for myelin production and understanding the mechanism of its delivery to oligodendrocytes emerges as a critical step in deciphering the regulatory pathway of iron homeostasis in the brain. Although transferrin is considered as the primary iron delivery agent to most cells in vertebrates, mature oligodendrocytes do not express TfR1 and take up iron by other mechanisms (Zhang *et al.* 2006). TfR1 may be expressed in oligodendrocyte progenitors in culture. However, during maturation TfR1 expression appears to downregulate in oligodendrocytes. Even in conditions of iron deficiency sufficient to trigger upregulation of TfR1 in other cells in the brain, TfR1 is not

detected in mature oligodendrocytes in adult brain (Hill *et al.* 1985, Hulet *et al.* 1999, Han *et al.* 2003).

During brain development, iron staining is first detected in microglia in the form of ferritin, but as the brain matures, ferritin staining is shifted from microglia to oligodendrocytes suggesting ferritin as a source of iron for oligodendrocytes (Connor *et al.* 1995a, Qi *et al.* 1995, Sanyal *et al.* 1996, Zhang et al. 2006). Indeed, in rodents, microglia respond to increased iron levels by expression and secretion of H-ferritin, which is then taken up by oligodendrocytes, promoting growth and preventing cytokine-induced cytotoxicity (Zhang et al. 2006). However, high iron levels increase cytokine release from activated microglia, leading to increased cytotoxicity of oligodendrocytes, which are more sensitive to iron toxicity and oxidative stress than microglia (Zhang et al. 2006). Additional evidence of the central role of microglia in iron homeostasis arise from animal models of hypoxia/ischemia showing iron-enriched microglia in damaged areas of the brain (Bidmon *et al.* 2001, Cheepsunthorn *et al.* 2001) and by the observation of iron enrichment in microglia when oligodendrocyte development is impaired, (Connor *et al.* 1990).

Tim2 (T-cell immunoglobulin mucin domain 2 protein) receptors were identified as the specific receptors mediating ferritin uptake and endocytosis in murine oligodendrocytes (Todorich et al. 2008). Tim2 is specific for H-ferritin and not L-ferritin, and its expression was demonstrated in the brain of rodents and was proposed as the major source of iron for oligodendrocytes (Todorich et al. 2008, Todorich *et al.* 2011). However, Tim2 was identified only in rodent and evidence for the existence of a Tim2 analogue in humans is still lacking (Kuchroo *et al.* 2003). To date, the only H-ferritin receptor identified in human cells is TfR1, which has been shown to bind both human transferrin and human H-ferritin but not murine H-ferritin (Li *et al.* 2010).

Ferritin as the major iron-binding protein

Ferritin in mammals is a mostly cytosolic protein of 450 kDa composed of a mixture of 24 subunits of heavy chain ferritin (H-ferritin) and light chain ferritin (L-ferritin). The H subunit of ferritin possesses ferroxidase activity allowing the oxidation of ferrous iron to ferric iron in the ferroxidase centre of ferritin, while the L subunit facilitates iron nucleation within the central core and promotes iron storage. Together the H and L subunits form a spherical protein that can store 4500 ferric iron atoms in a bioavailable and non-toxic form as the mineral ferrihydrite (Arosio *et al.* 2009, Arosio & Levi 2010). Hemosiderin is a degradation

product of ferritin representing an insoluble and bio-unavailable pool of iron often observed in areas of massive cell death. The ratio of H to L-subunits within ferritin varies among different organs and within cells of the same tissue (Connor *et al.* 1994). Expression of ferritin is regulated at the translational level by IRPs, which may lead to an 80-fold increase in ferritin levels while degradation is triggered by certain iron chelators and by lysosomal activity (Pietsch *et al.* 2003, Hintze & Theil 2005). Although ferritin is considered primary as an intracellular iron storage protein, it is also present in circulating form in serum and CSF and there is growing evidence of additional functions beyond iron storage. Low serum ferritin levels correlates with iron depletion, while elevated serum ferritin results not only from elevated body iron stores, but also from inflammation in patients with normal body iron stores (Wei *et al.* 1990, Fahmy & Young 1993). Indeed, inflammation processes increase H-ferritin expression, which sequester iron and protect against oxidative stress, resulting in the anemia of chronic inflammation (Wessling-Resnick 2010). Also brain ferritin has a higher abundance of H subunits over the L subunits in comparison to serum ferritin suggesting extended functions of ferritin within the brain in comparison to the periphery, where ferritin is mostly used as a storage protein.

Hereditary ferritinopathy is an autosomal dominant condition caused by mutations in the L-ferritin gene that disrupts the iron storage ability of ferritin and promotes ferritin aggregation in association with iron, which occurs prominently in the nuclei of neurons, oligodendrocytes, microglial cells and the extracellular space (Cozzi *et al.* 2006, Muhoberac *et al.* 2011). Pathologically, the brain tissue of hereditary ferritinipathy cases shows, in addition to iron accumulation, elevated levels of neuroglobin, deposits of cytoglobin, and p53-mediated apoptosis of neurons and glia in specific regions, with increased lipid peroxidation and abnormal nitration of proteins (Mancuso *et al.* 2005, Powers 2006). However, little is known about ferritin binding, storage, secretion, transfer and regulation that may explain possible additional functions of ferritin within the brain and other tissues.

Regulation of brain iron through iron response proteins

Iron response proteins are ubiquitously expressed cytosolic proteins that alter their activities according to intracellular iron levels. In mammalian cells two IRPs exist, IRP1, which is expressed in kidney liver and brown fat, and IRP2, which is mainly expressed in the central nervous system (CNS) (Meyron-Holtz *et al.* 2004). As discussed in the section on peripheral iron homeostasis above, iron levels in the brain modulate the expression of iron-responsive proteins through the IRP/IRE machinery. In contrast to IRP2, IRP1 functions as IRE-binding

protein only at low iron concentrations, while at high iron concentrations, it functions as an aconitase through acquisition of an iron-sulfur cluster ligand (Zhang *et al.* 2014).

The importance of the IRP/IRE regulation is demonstrated by the embryonic lethality of the simultaneous deletion of both IRPs in mice, while mice with deletion of either IRP1 or IRP2 are viable and fertile, attesting for overlapping functions of both IRPs (Meyron-Holtz et al. 2004). However, phenotypic characterization of IRP1-/- and IRP2-/- mice revealed distinct functions of both IRPs. While IRP1 deletion in mice caused impairments of erythropoiesis and the pulmonary and cardiovascular systems (Ghosh *et al.* 2013), IRP2 deficiency resulted in mostly neurodegenerative symptoms caused by increased expression of ferritin and decrease in expression of the iron importer TfR1, leading to functional iron deficiency (LaVaute *et al.* 2001). Interestingly, activation of IRE-binding activity of IRP1 through oral treatment with Tempol -a scavenger of reactive oxygen species, which disrupts the iron-sulfur cluster ligand of IRP1- improved the neurodegenerative symptoms of IRP2 deficient mice (Ghosh *et al.* 2008, Wilcox & Pearlman 2008). In contrast, deletion of one or more copies of IRP1 in IRP2 deficient mice exacerbated the neurodegenerative symptoms confirming the causative role of iron dysregulation in IRP-mediated neurodegeneration (Zhang et al. 2014). Although patients with IRP2-mediated diseases have not been identified yet, assessment of ferritin and transferrin receptor expression levels in different human neurodegenerative diseases might be helpful in identifying pathological conditions related to IRP2 deficiency, which can be treated with IRP1-activating substances such as Tempol and compensate for the loss of function of IRP2.

Altered iron homeostasis in neurodegenerative diseases

The requirement of iron for proper brain development, neurogenesis and cognitive functions was recognized by investigating patients suffering from iron deficiency, which is the most common nutrient deficiency worldwide (McLean *et al.* 2009). Accordingly, patients suffering either from prenatal or postnatal iron deficiency show persistent deficits in neurodevelopment including impairment in learning and memory (Radlowski & Johnson 2013). Normal aging is accompanied by progressive iron accumulation in the brain and is most evident within brain regions like the substantia nigra, putamen, globus pallidus, caudate nucleus and cortices, which have been all associated with neurodegenerative disorders (Berg & Youdim 2006, Rodrigue *et al.* 2011, Callaghan *et al.* 2014, Ward et al. 2014, Hare *et al.* 2015a).

A stronger correlation between iron and neurodegeneration has been significantly acquired through the investigation of a group of diseases known as Neurodegeneration with Brain Iron Accumulation (NBIA), which are characterized by excessive iron deposition in the basal ganglia, globus pallidus, SN and the cerebellar dentate nuclei (Kurian *et al.* 2011, Schneider & Bhatia 2013). Although iron accumulation is a common event in all NBIA, it is interesting to note that among all 10 identified forms of NBIA, only two forms are linked to mutations in genes directly involved in iron metabolism: neuroferritinopathy and aceruloplasminemia, which are caused by defects in the L-ferritin and ceruloplasmin genes, respectively, while the other NBIA forms are involved in several pathways including lipid metabolism, mitochondrial function, lysosomes and autophagosomes (Singh et al. 2014). However, defects in iron homeostasis spread beyond the NBIA disorders and several studies have shown that iron is implicated in several other neurodegenerative diseases such as Alzheimer´s, Parkinson´s, Huntington´s, amyotrophic lateral sclerosis (ALS) and prion diseases. Intracellular iron has been recently implicated in a specific cell death pathway termed ferroptosis, which is independent from its capacity to trigger ROS production and was related to one or more iron-dependent enzymes functioning as part of the core oxidative lethal mechanism (Dixon *et al.* 2012). Indeed, the iron-dependent cell death pathway implicated in ferroptosis was identified as a specific and regulated cell death pathway, which is different from apoptosis, necrosis and autophagy (Dixon & Stockwell 2014, Galluzzi *et al.* 2015, Jiang *et al.* 2015). Ferroptosis can be triggered by structurally diverse small molecules such as erastin, sulfalazine and RSL3 and can be prevented by lipophilic antioxidants, such as trolox and vitamin E, and by iron chelators such as deferoxamine, and Ferrostatin-1 was the first identified ferroptosis inhibitor in cancer cells (Dixon et al. 2012, Dixon & Stockwell 2014). The precise role of iron in ferroptosis is not clear but it is hypothesized that glutathione depletion leads to the iron-dependent accumulation of ROS, especially lipid ROS, which are sufficient to induce cell death (Dixon et al. 2012). Recently, lipid peroxidation through inhibition of glutathione peroxidase 4 (Gpx4) has been identified as a cause of a pathologically relevant form of ferroptosis, and lead to the discovery of Liproxstatin-1 as a potent inhibitor of ferroptosis in cells, *Gpx4* knockout mice, and in a pre-clinical model of ischaemia/reperfusion-induced hepatic damage (Friedmann Angeli *et al.* 2014). Thus, suggesting inhibition of the ferroptosis pathway as a possible mechanism by which iron chelation therapies may prevent neuronal cell death. Together, these data suggest a direct relationship between iron homeostasis and the pathomechanism involved in several

neurodegenerative disorders and next we discuss evidences for a role of iron in two of the most prevalent neurodegenerative diseases in the elderly: AD and PD.

Alzheimer´s disease

AD is a slowly progressive neurodegenerative disorder involving cortical and hippocampal neuronal loss leading to impairment in cognition and profound memory loss. AD is the leading cause of dementia in aging and is characterized by two pathological hallmarks: the accumulation of aggregated amyloid beta peptide (Aβ), the major constituent of extra neuronal senile plaques (SPs), and excessively phosphorylated microtubule-associated protein tau, which forms intracellular neurofibrillary tangles (NFTs). The amyloid theory, which has dominated AD research over the last three decades links disease pathomechanism solely to the formation, accumulation and toxicity of the Aβ peptides. Consequently, research and several clinical trials focused on developing treatment strategies targeting removal of this peptide from the brain and all have failed so far in attenuating disease progression, even if Aβ clearance was achieved (Morris *et al.* 2014). There is little doubt that aggregated Aβ plays a central role in the pathology of AD. However, it is evident from the current literature that this peptide alone does not explain the pathological progression of the disease and the disease is believed to involve several pathways including dysregulated metal homeostasis, impaired glial functions, inflammation, mitochondrial dysfunction and oxidative stress, which have been relatively neglected over recent years (Bush & Curtain 2008, von Bernhardi & Eugenin 2012).

The discovery of apolipoprotein E (apoE) E4 allele as the strongest genetic risk factor for the development of AD and its gene dosage effect on the age of onset of the disease has focussed attention to the importance of apoE in neurobiology and neurodegenerative diseases (Corder *et al.* 1993, Saunders *et al.* 1993, Roses 1996). In humans, apoE exists in three different isoforms designated E2, E3 and E4, with E3 being considered the normal and most common isoform (allelic frequency: E3, 77%; E4, 15%; E2, 8%) and differ from E4 and E2 by Cys/Arg substitutions at residue 112 and 158 respectively (Huang & Mahley 2014). ApoE is a 34 kDa protein associated with very-low-density lipoproteins (VDL) and high-density lipoproteins (HDL), which was first identified for its role in cholesterol metabolism. ApoE binds to specific cell-surface receptors including the LDL receptor family and heparin sulfate proteoglycans (HSPG) and plays a crucial role in the homeostatic control of plasma and tissue lipid content (Huang & Mahley 2014). ApoE is also highly expressed in the brain and constitutes the principal lipid transport system in CSF, and plays a crucial role in repair by

redistributing lipids to regenerating axons and to Schwann cells during remyelination (Huang & Mahley 2014). In the brain, apoE is primary expressed by astrocytes with lower expression in microglia while neurons does not express apoE under normal conditions (Xu *et al.* 2006). However, kainic acid treatment induces intense expression in injured neurons, demonstrating apoE expression in neurons in response to excitotoxic injury (Xu et al. 2006). Despite several biochemical, cell biological and transgenic animal studies, the underlying mechanisms associating apoE4 with AD pathogenesis are still under debate (Huang & Mahley 2014).

Involvement of iron in AD pathology

Elevated iron in AD brains was first reported in 1953 and its association with senile plaques and NFTs or with ferritin in the surrounding glia cells was confirmed in several studies (Goodman 1953, Connor et al. 1992b, Smith *et al.* 1997, Lovell *et al.* 1998, Smith *et al.* 2007). However, iron accumulation is not only restricted to senile plaques and NFTs. Advances in iron quantification techniques, especially magnetic resonance imaging (MRI) methods, demonstrated that brain iron increases with aging, a process that is enhanced and localized to restricted regions in AD brain such as the parietal cortex, motor cortex and hippocampus (Bartzokis *et al.* 1994, Bartzokis & Tishler 2000, Ding *et al.* 2009, Pfefferbaum *et al.* 2009, Bilgic *et al.* 2012, Luo *et al.* 2013, Ghadery *et al.* 2014, Langkammer *et al.* 2014, Tao *et al.* 2014). Recently, application of specialized techniques such as size exclusion chromatography-inductively coupled plasma-mass spectrometry (SEC-ICP-MS), which enables measurement of iron saturation of proteins such as ferritin or transferrin rather than measurement of total protein levels (Hare *et al.* 2013b), revealed significantly decreased plasma iron in AD patients (Faux *et al.* 2014) due to transferrin desaturation (Hare *et al.* 2015b). Recently, another study showed significantly decreased expression of aconitase 1 (ACO1), CP and APP in peripheral blood mononuclear cells in AD patients as compared to controls, pointing to a downregulation of cellular iron export in AD over control (Guerreiro *et al.* 2015).

Aβ is formed under normal physiological conditions and its neurotoxicity is dependent on the oligomeric state and the length of the Aβ peptide, in addition to its concentration. Accordingly, Aβ (1-42) peptides are more toxic than the Aβ (1-40) peptides and are more insoluble, being the most common species in amyloid plaques. Several studies showed that iron promotes the aggregation, oligomerization and amyloidosis of Aβ peptides and that the iron complexed with Aβ peptides is toxic in cultured cells (Mantyh *et al.* 1993, Schubert & Chevion 1995, Huang *et al.* 2004, Liu *et al.* 2011). Current literature acknowledges the likely

contribution of metals bound to Aβ to it neurotoxic effect. However, several studies suggest that the role of metals in general is beyond a simple contribution to Aβ toxicity. Indeed, recent results suggest that Aβ may not be toxic in the absence of redox metals and that metals are needed for its aggregation. Accordingly, the oxidative damage associated with Aβ is rather due to its high affinity for binding iron (and copper) and its capacity to chemically reduce both metals leading to catalytic hydrogen peroxide formation and subsequent oxidative damage (Huang *et al.* 1999, Jomova *et al.* 2010).

In addition to Aβ peptides, iron also binds to tau, affects its phosphorylation and induces aggregation of hyperphosphorylated tau (Fig. 3), which can be reversed using iron chelators (Yamamoto *et al.* 2002, Lovell *et al.* 2004, Chan & Shea 2006, Amit *et al.* 2008). Tau accumulation in NFTs is also associated with an increased induction of HO-1 (Schipper *et al.* 2006, Wang *et al.* 2015), which is a potent antioxidant through its function in metabolizing haem released from damaged mitochondria. But HO-1 also contributes to ferrous iron release, which might trigger additional oxidative stress through radical formation (Perry *et al.* 2002, Ward et al. 2014). An important consideration in discussing the levels of iron in AD is to emphasize that an increase in total brain iron is not, alone, necessary to induce oxidative stress in the brain but, rather, an imbalance in homeostasis may have a dual effect by inducing an iron-rich environment favouring oxidative stress and cell death around amyloid plaques and NFTs, while other brain areas may be deprived of iron leading to impaired neuronal function.

An important link between iron homeostasis and AD pathology is the discovery that translation of the amyloid precursor protein APP is directly regulated by cellular iron levels through IREs identified in the 5´-UTR mRNA, thus defining APP as a metalloprotein (Rogers *et al.* 1999, Rogers *et al.* 2002). APP is a transmembrane protein that has been almost exclusively studied for its cleavage product Aβ, while its primary function is still not understood and a general involvement in synapse formation, neuronal plasticity and ion transport have been described (revised in (Caldwell *et al.* 2013)). Under normal conditions, most neuronal APP undergoes proteolytic cleavage through the non-amyloidogenic pathway, which involves the action of α-secretase followed by the γ-secretase. Aβ peptides are formed through the amyloidogenic pathway, which requires cleavage of APP first by β-secretase and then γ-secretase. Because iron levels modulate APP expression at the translational level, increased iron concentrations in neurons increase the amount of APP protein that can undergo amyloidogenic processing and potentially increase Aβ production. Early studies have also pointed to a modulatory effect of iron on the α-secretase cleavage of APP (Bodovitz *et al.*

1995). Moreover, the proteolytic activation of the inactive forms of α-secretase and β-secretase is modulated by furin, which is promoted by iron at the transcriptional level (Silvestri & Camaschella 2008). Accordingly, excess iron lead to decreased furin protein concentrations, which favors β-secretase activity thereby stimulating the amyloidogenic pathway and Aβ production (Silvestri & Camaschella 2008, Ward et al. 2014). APP has been also shown to facilitate iron efflux from neurons through its stabilizing interaction with surface ferroportin, and APP depletion in cultured neurons and mouse models leads to intracellular iron retention (Duce *et al.* 2010, Wan *et al.* 2012, McCarthy et al. 2014, Wong *et al.* 2014b). Tau deficiency induces intracellular iron accumulation and dopaminergic neuronal loss leading to parkinsonism and dementia, which was due to impaired APP trafficking to the membrane, thus preventing its interaction with ferroportin and suppressing iron efflux from neurons (Fig. 3) (Lei *et al.* 2012).

Further supporting evidence for a link between brain iron metabolism and AD pathology arose from a recent longitudinal study in AD patients and healthy individuals, which showed that CSF ferritin levels were negatively associated with cognitive performance over seven years and predicted the conversion from mild cognitive impairment to AD (Ayton *et al.* 2015a). Moreover, the study showed a robust correlation between ferritin and apoE protein levels, and an elevation of ferritin by the AD risk allele *APOE E4*, implicating apoE protein in brain iron metabolism (Ayton et al. 2015a). This finding suggests that ferritin may reflect the mechanism by which *APOE E4* is a risk factor for AD. Future studies will be required to investigate the relationship of apoE and ferritin in AD pathogenesis.

Parkinson´s disease

PD is the second most prevalent age-related neurodegenerative disorder affecting 1-2% of the population over the age of 65. Clinically, PD patients are characterized by motor dysfunction, rigidity, bradykinesia, which are accompanied by cognitive decline and dementia at advanced stage of the disease. Pathologically, the disease is characterized by the loss of dopaminergic neurons in SN pars compacta and the accumulation in neurons of aggregated α-synuclein protein in inclusions called lewy bodies (Fearnley & Lees 1991, Lotharius & Brundin 2002, Moore *et al.* 2005). Most cases of PD (90%) are sporadic with several risk factors, while the familial cases (10%) are linked mainly to mutations in α-synuclein in addition to other several genes implicated in mitochondrial function, ubiquitination and autophagy (Polymeropoulos *et al.* 1997, Kitada *et al.* 1998, Bonifati *et al.* 2003, Valente *et al.* 2004, Nuytemans *et al.* 2010). Although the function of α-synuclein is not fully characterized, its overexpression is sufficient

to cause PD implicating this protein as a hallmark in the pathology of the disease (Decressac *et al.* 2012).

With the continuous development in imaging methods that are able to measure iron in specific regions of the brain, the accumulation of iron in neurons and glia of the SN in PD patients has been firmly demonstrated and several studies showed that iron concentrations in SN of PD patients also correlate with disease severity (Dexter et al. 1987, Dexter *et al.* 1989, Hirsch *et al.* 1991, Pyatigorskaya *et al.* 2015). Moreover, iron has been also shown to induce the conversion of α-synuclein from the α-helix to the β-sheet conformation characteristic of the Lewy bodies present in SN of PD patients (Uversky *et al.* 2001, el-Agnaf & Irvine 2002). However, iron increase alone in SN cannot account for the specific cell death observed in SN pars compacta. Indeed, several early studies showed that iron is not equally distributed within the different areas of the SN (Sofic *et al.* 1988, Sofic *et al.* 1991, Loeffler *et al.* 1995, Berg *et al.* 1999, Griffiths *et al.* 1999, Faucheux *et al.* 2002, Atasoy *et al.* 2004, Snyder & Connor 2009). However, recent analyses applying imaging techniques such as laser ablation-inductively coupled plasma mass spectrometry (LA-ICP-MS) and immunohistochemical methods revealed that the mouse SN pars compacta contains 25 % less iron than the adjacent SN pars reticulata and identified the overlap of iron and dopamine as a high risk factor for the selective cell death in the SN pars compacta (Hare *et al.* 2014). Accordingly, the study attributed the vulnerability of the SN pars compacta to neurodegeneration to the exceptional microscopic colocalization iron and dopamine within this subregion, while isolated high basal iron (in the SN pars reticulata) or high dopamine concentrations (in the ventral tegmental area) explain why these subregions are relatively spared in Parkinson's disease (Hare et al. 2014). The presence of neuromelanin in neurons of the SN pars compacta represent an additional risk factor, which due to its high affinity for iron may further exacerbate the vulnerability of this brain region to oxidative stress (Enochs *et al.* 1994, Zecca *et al.* 1994, Zucca *et al.* 2014).

Beside the elevation of iron in the SN and its involvement in α-synuclein aggregation, the association between iron homeostasis and PD appears to be much more pronounced and alterations in several key proteins of iron metabolism are continuously reported (Fig. 3). Indeed, in addition to high iron loading, decreased ferritin levels have been reported in the SN of PD patients. Specifically, H ferritin and L ferritin were reported to be 75% and 37% of normal, respectively (Dexter *et al.* 1991, Connor et al. 1995b). Decreased ferritin levels may be explained by the lack of regulation, which correlates with the sustained IRP1 activity that

has been reported in the SN of post-mortem PD brains (Faucheux et al. 2002). Upregulation of DMT1 and reduced ferroxidase activity of CP, which would lead to increased intracellular iron levels, have been also reported in PD patients and PD animal models (Boll *et al.* 1999, Salazar *et al.* 2008, Olivieri *et al.* 2011, Ayton *et al.* 2013). Serum iron levels have been also proposed as a possible marker for PD in a large Mendelian randomization study (Pichler *et al.* 2013). A link between AD, PD and iron has been also established by characterizing tau knockout mice. Accordingly, loss of function of tau and the resulting impaired iron export through APP resulted in age-dependent brain atrophy, iron accumulation and neuronal loss in SN, cognitive deficits and parkinsonism, which were prevented using iron chelation (Lei *et al.* 2010, Lei et al. 2012, Lei *et al.* 2014). Recently, another study showed that APP expression is decreased in SN dopaminergic neurons of human PD and that APP knockout mice develop iron-dependent neuronal cell death in the SN (Ayton *et al.* 2015c). Accordingly, APP reduced expression was attributed to NO-induced suppression of APP translation by disrupting IRP2. This study connected NO to iron metabolism and indicated how nitrosative stress may act as an upstream event in the pathology of PD as previously reported (Przedborski *et al.* 1996, Ryan *et al.* 2013). Recently, a similar mechanism involving NO-mediated disruption of iron metabolism was reported in hypoxia-ischemia-mediated neonatal brain injury (Lu *et al.* 2015).

Hemochromatosis: a counterargument?

The debate in considering iron as a cause or consequence of major neurodegenerative disorders is partially justified by the fact that neurological symptoms are not major characteristics of diseases such as hemochromatosis, which increases iron levels in the body. Hereditary hemochromatosis is a recessive autosomal disorder characterized by an increased absorption of dietary iron and a subsequent overdeposition in tissues and organs of the periphery. The mutation H63D in the HFE gene was extensively investigated as a potential risk factor for neurodegenerative diseases due to its prevalence (as high as 24%) in Caucasians (Burt *et al.* 1998, Adams *et al.* 2005). However, different studies analysing the frequency of this mutation in neurodegenerative disorders showed different results: an increase in AD (Moalem *et al.* 2000, Sampietro *et al.* 2001), no association with AD (Candore *et al.* 2003) or a possible association with PD (Guerreiro *et al.* 2006). Interestingly, several studies showed a correlation between the H63D-HFE mutation and the AD risk allele *APOE E4* and a significant increase in early onset of AD when both proteins were present (Combarros *et al.* 2003, Pulliam *et al.* 2003, Percy *et al.* 2008). Thus, raising the question whether disruption of iron and cholesterol metabolism are interconnected in AD. This

hypothesis was supported in a recent study, which showed that expression of HFE variants in mice resulted in reduction of brain cholesterol levels, which was accompanied by impairment in memory and learning (Ali-Rahmani *et al.* 2014). Additional studies will be required to investigate the relationship between iron and cholesterol metabolism in neurodegenerative disorders.

Iron chelation as a therapeutic approach for AD and PD

Alzheimer´s disease

Aging is the highest risk factor for AD with an incidence of 50% in people over the age of 85 (Perls 2004). Over the last 20 years, most of the research in AD therapy targeted removal of amyloid using passive or active immunotherapy or impairing amyloid production using secretase inhibitors. Although many studies have shown promising results in AD mouse models, failures in human clinical trials are continuously reported raising the fundamental question on how representative amyloid burden is for the dementia process (Morris et al. 2014). Indeed, several studies showed that amyloid accumulation as measured by positron emission tomography (PET) imaging is observed also in cognitively normal individuals and does not correlate with disease severity in AD (Engler *et al.* 2006, Price *et al.* 2009, Chetelat *et al.* 2013). Thus, raising the question whether amyloid accumulation is associated with normal aging rather than exclusively with AD (Rentz *et al.* 2010). It should be mentioned that the FDA has approved only five drugs for AD over the last years and none of them favours amyloid hypothesis (Morris et al. 2014).

Given the evidence of pathological iron accumulation in AD, iron removal from certain brain regions without causing a systematic iron deficiency should be taken into consideration (Fig. 4) (Bush 2008, Ayton *et al.* 2015b). In 1991, a single blind study in 48 AD patients over a period of 24 months have explored the therapeutic efficacy of iron chelation using intramuscular injections of the iron chelator desferrioxamine (Crapper McLachlan *et al.* 1991). The study showed a significant reduction in cognitive decline in the desferrioxamine-treated group as compared to patients given placebo (Crapper McLachlan et al. 1991). Despite the promising results, no other clinical trials using desferrioxamine have been reported since 1991. Later, treatment with 5-Chloro-7-iodo-quinolin-8-ol (clioquinol), which is a moderate chelator of iron, copper and zinc was found to reduce amyloid deposition in the brain and prevent memory impairment in an AD animal model (Cherny *et al.* 2001, Grossi *et al.* 2009) and showed promising results in a pilot phase 2 clinical trial (Ritchie *et al.* 2003). PBT2,

another chelator derivative of clioquinol has been shown to promote amyloid plaque degradation and relocating excess released metals to other depleted neuronal compartments (Adlard *et al.* 2008, Adlard *et al.* 2011, Crouch *et al.* 2011). Iron chelation using intranasal deferoxamine (DFO) has been tested in several studies and was shown to reverse iron-induced memory deficits, inhibits amyloidogenic APP processing and Aβ aggregation in mouse models of AD (Fine *et al.* 2012, Guo *et al.* 2013, Guo *et al.* 2015) and improve motoric deficits and pathology in the α-synuclein PD mouse model (Febbraro *et al.* 2013).

Parkinson´s disease

The efficacy of iron chelation in PD has been investigated using either genetic or pharmacological methods and was shown to reduce iron levels and prevent toxicity in the MPTP mouse model of PD (Kaur *et al.* 2003). Difficulties in using iron chelation are largely due to the inability of large molecules such as desferrioxamine to penetrate the BBB, which is a prerequisite to remove iron from the brain. However, the risk of peripheral iron depletion from plasma should be taking into account, as several neurodegenerative disorders, which showed dysregulated brain iron levels, did not reveal any abnormalities in peripheral iron levels (Fig. 4). Therefore, several low molecular compounds with varying affinities for iron have been developed such as clioquinol, PBT2 and deferiprone with potential for the treatment of several neurodegenerative disorders (Weinreb *et al.* 2013). Recently, clioquinol has been shown to rescue Parkinsonism and dementia phenotypes of the tau knockout mouse (Lei et al. 2012, Lei *et al.* 2015).

Deferiprone has been already clinically approved for the treatment of the iron overload disorder thalassemia and was also tested in the NBIA neurodegenerative disorder Friedreich´s ataxia (Boddaert *et al.* 2007). In 2012, Kwiatkowski and colleagues investigated the efficacy of deferiprone in a single patient with mild Parkinsonism who showed elevated iron levels in the bilateral dentate nuclei, SN and red nuclei as measured with T2*-weighted brain magnetic resonance imaging (T2*-MRI) (Kwiatkowski *et al.* 2012). Accordingly, long-term treatment of the patient with deferiprone showed a rapid decrease of iron in the bilateral dentate nuclei with a milder, delayed but sustained reduction in the SN and a positive improvement of the patient condition was recorded after one year of treatment using the unified PD rating scale with no apparent neurological or haematological side effects (Kwiatkowski et al. 2012). Devos and colleagues recently reported a double-blind, randomized, placebo-controlled clinical trial of early-stage PD patients over 12 months treated with deferiprone (30 mg/kg/day) (Devos *et al.* 2014). Results revealed that patients who received deferiprone

showed decreased motor handicap progression (measured by unified PD rating scale) and decreased iron deposits in the SN (measured by T2*-MRI) (Devos et al. 2014). Deferiprone may be the first disease-modifying treatment for PD and currently is being tested further in a phase 3 clinical trial for PD patients (clinical trial NCT01539837).

Conclusions and future perspectives

AD and PD are the most common neurodegenerative diseases in the elderly. Despite differences in clinical presentation and pathologies, both diseases may involve similar mechanisms linking oxidative stress, protein aggregation, and mitochondrial dysfunction. Extensive investigation of iron homeostasis and the recent advance in iron imaging methods clearly points to a role for iron in the pathology of neurodegenerative diseases. Beyond AD and PD, iron is implicated in several other diseases such as Huntington´s, ALS and prion diseases. Brain iron metabolism is largely unaffected by peripheral changes in iron levels, supported by the fact that diseases characterized by increased peripheral iron such as hemochromatosis and thalassemia do not impact on brain iron levels nor do they increase the incidence of neurodegeneration. Increase in total brain iron can trigger neurodegeneration as observed in NBIA disorders. However, due to the highly reactive character of iron, its heterogeneous distribution and enrichment in certain brain areas, a local dysregulation in brain iron homeostasis is sufficient to trigger a cascade of events leading to oxidative stress or ferroptosis in particular brain regions, while depleting iron from other cellular compartments resulting in neuronal cell death. Therefore, future challenges are required in developing methods that are able to quantitatively measure iron in different areas of the brain in different neurological disorders. Although, iron dysregulation is implicated in several neurodegenerative diseases, the mechanisms involved are still poorly understood and future research is required to gain a better understanding of brain iron homeostasis and elucidate the factors and mechanisms involved in its dysregulation. The recent discovery of ferroptosis as an iron-dependent cell death pathway opens new possibilities for pharmacological interventions and future progress in the development of ferroptosis inhibitors that can cross the BBB will be required to evaluate the potential of ferroptosis inhibition in the development of future therapies.

ARRIVE guidelines have been followed:
Yes
=> if No, skip complete sentence
=> if Yes, insert "All experiments were conducted in compliance with the ARRIVE guidelines."

Conflicts of interest: AIB is a shareholder in Prana Biotechnology Pty Ltd., Cogstate Pty Ltd, Eucalyptus Pty Ltd., Mesoblast Pty Ltd., Brighton Biotech, LLC, and a payed consultant for Collaborative Medicinal Developments LLC and Brighton Biotech, LLC. AAB has no competing interests to declare.
=> if 'none', insert "The authors have no conflict of interest to declare."
=> otherwise insert info unless it is already included

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Figure legends

Figure 1. Systemic iron homeostasis.

Duodenal enterocytes absorb iron as ferrous, ferric and heme iron. Ferric iron must be first reduced to ferrous iron by duodenal cytochrome b (Dcytb) and then transported across the apical membrane of the enterocyte by the divalent metal transporter-1 (DMT1). Heme is taken up via heme carrier protein 1 (HCP-1) and is subsequently degraded by heme oxygenase 1 (HO-1) to release iron. The absorbed iron constitutes the labile iron pool (LIP) of the enterocyte, which can be either stored in ferritin or transported across the basolateral membrane via ferroportin (Fpn), which requires the oxidation of ferrous iron into the ferric form by the ferroxidase hephaestin. Ferric iron is finally bound by plasma transferrin (Tf) and delivered to the different tissues through binding of Tf to the transferrin receptor 1 (TfR1) complex. Hepatocytes monitor iron status of the body by sensing Tf saturation in plasma via TfR1, transferrin receptor 2 (TfR2) and the HFE protein and respond by regulating the expression of the hormone hepcidin. Accordingly, iron overload induces expression and release of hepcidin that binds to Fpn and triggers its degradation in the enterocyte (green arrows). In contrast, iron deficiency inhibits hepcidin expression and restores normal iron absorption from the enterocyte.

Figure 2. Brain iron metabolism.

Iron import into the brain starts by binding of circulating transferrin (Tf) to transferrin receptor-1 (TfR1) located at the apical membrane of brain capillary endothelial cells (BCEC). Based on the presence or absence of divalent metal transporter-1 (DMT1) in BCECs, two different transport models have been proposed. In the transcytosis model (BCECs, red arrows), the transferrin-receptor complex is transported into endosomes across the BCECs cytosol and is directly released into the brain at the abluminal membrane of BCECs, while the receptor-mediated endocytosis model (BCECs, blue arrows) proposes that subsequent to transferrin-receptor complex internalisation, iron is released from endosomes in BCECs cytosol via DMT1 and exported into the brain through ferroportin. Iron import into astrocytes is also not clear and possibly iron is taken up via DMT1 or other transporters. Astrocytes are also proposed to release low molecular compounds such as ascorbate, citrate and ATP, which bind iron released from BCECs and contribute to the non-transferrin bound iron (NTBI) pool of the brain. The location of astrocytes in close proximity to BCECs suggests an important role in distributing iron to other cells in the CNS. Iron can be stored in ferritin or exported from astrocytes via ferroportin (Fpn) and a physically associated ceruloplasmin (CP). The ferrous iron presented by ferroportin is catalytically oxidized by CP and inserted into extracellular Tf, which is secreted from the choroid plexus. Transferrin is the major iron transport protein of the brain, which is taken up by neurons through TfR1-meditaed endocytosis and internalization in endosomes. Upon acidification in endosomes, iron is released from Tf, reduced to the ferrous form and exported into the cytosol by DMT1. Thus, contributing to the labile iron pool (LIP) of neurons. Iron export from neurons is carried out by Fpn and circulating CP and requires Fpn stabilization by amyloid precursor protein (APP), which is transported to the membrane by the microtubule-associated protein tau. Microglia has been shown to take up iron and export ferritin although the mechanisms involved are poorly understood. Up to date, only oligodendrocytes were reported to import extracellular ferritin through specific receptors (Tim2), while TfR1 expression is observed only during maturation. Oligodendrocytes require iron also for myelin synthesis and formation of the myelin sheath, which surrounds neurons.

Figure 3. Brain iron dysregulation in AD and PD.

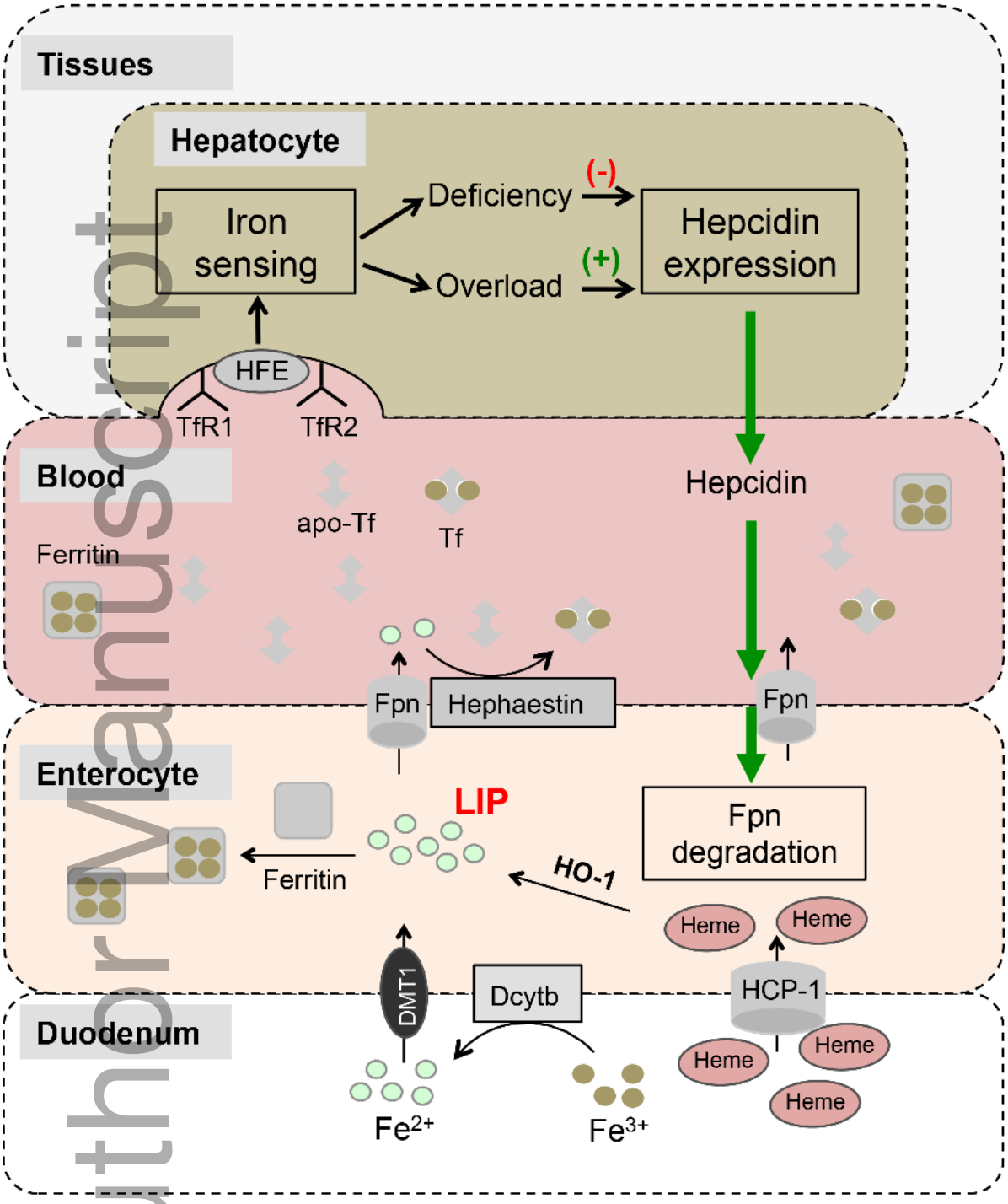
Proposed sequence of biochemical events in healthy individuals (middle panel), AD (right panel) and PD (left panel) in response to an increase in labile iron pool (LIP) of neurons as observed in healthy aging. In healthy individuals, neuronal cells respond to an increase in LIP

by regulating proteins responsible for iron uptake, storage and excretion via iron response proteins (IRP1/2). Accordingly, increased LIP results in an increased expression of ferritin and amyloid precursor protein (APP), promoting iron storage and export, respectively, while iron import is reduced through impairing the expression of transferrin receptor-1 (TfR1) and divalent metal transporter-1 (DMT1). In PD, a decrease in CP activity and a sustained IRP1 activity, which was reported in post-mortem PD brains fails to regulate the expression of ferritin, APP, DMT1 and may promote the increase in LIP in neurons of the SN. Nitric oxide (NO) was also implicated in IRP1/2 dysregulation in PD. In addition, tau deficiency is sufficient to induce Parkinsonism in mice through an impaired membrane trafficking of APP and consequently a reduced iron export capacity through ferroportin (Fpn) and ceruloplasmin (CP). Increased iron in PD can further trigger α-synuclein aggregation and the presence of dopamine in neurons of the SN represents an additional synergistic risk factor for the formation of reactive oxygen species (ROS) and oxidative stress. In AD, tau hyperphosphorylation and subsequent aggregation into neurofibrillary tangles (NFTs) contributes to reduce the iron export capacity of neurons in response to an increased LIP by reducing the pool of soluble tau. In addition, AD is characterized by an increase in APP cleavage into Aβ, which through its high affinity to metals aggregates into amyloid plaques and contributes to synaptic loss and neuronal death. It should be noted that iron accumulation in neurons of AD and PD may lead to iron depletion in other cells of the brain and contribute to neurodegeneration. Hallmarks of dysregulated iron metabolism in AD and PD are highlighted in red and arrows represent changes in response to an increase in LIP under healthy conditions (green) or AD and PD (red).

Figure 4. The prevention of iron-dependent cell death.

Iron is required for the synthesis of essential proteins such as neuromelanin and heme, while excess iron is stored in ferritin and represents a safe and biodegradable form of iron. The iron status of the cell is determined by the cytosolic labile iron pool (LIP), which is a term used to define chelatable complexes of iron that are easily accessible to fulfil the metabolic requirement of the cell and are also used as an iron sensor, which determine the expression of proteins of the iron uptake, storage and export via the IRP/IRE machinery. The concept of iron chelation therapy is to attenuate the susceptibility of iron for radical formation and induction of oxidative stress without generating a long term local or systemic iron deficiency. Increase in intracellular iron levels is also linked to the induction of a specific non-apoptotic cell death pathway termed ferroptosis. Therefore, iron chelators in neurodegenerative

disorders are designed to cross the BBB and coordinate metals in a safe form with an effective binding affinity for the LIP. Thus, allowing iron reduction in iron-overloaded cellular compartments but also ensuring a redistribution of iron to major transport proteins such as transferrin.



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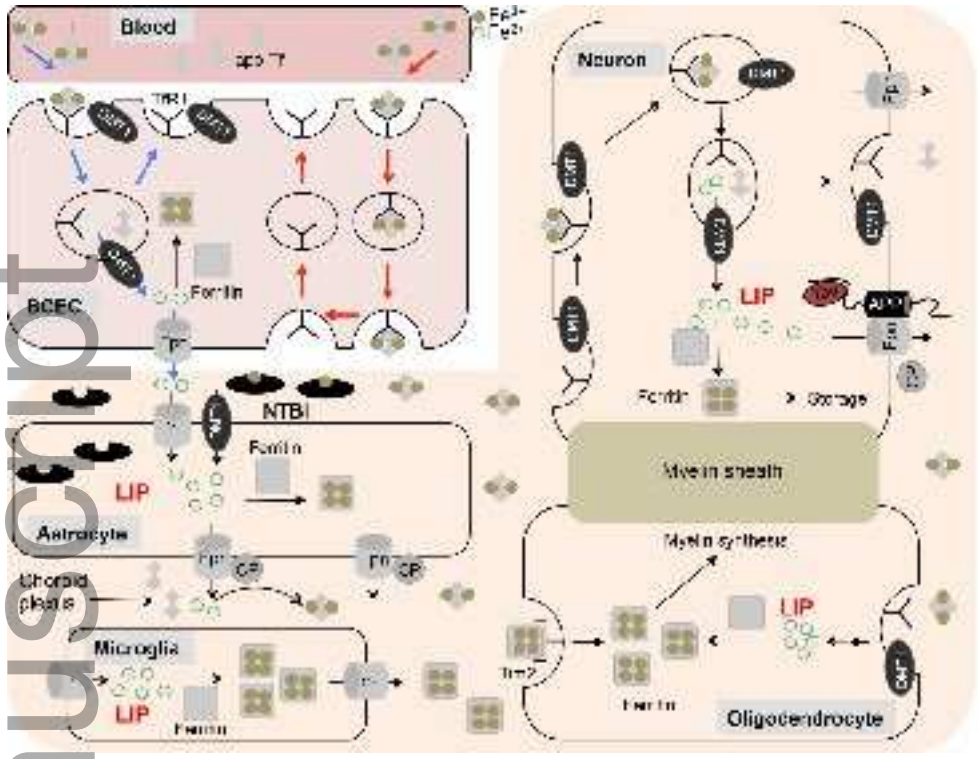

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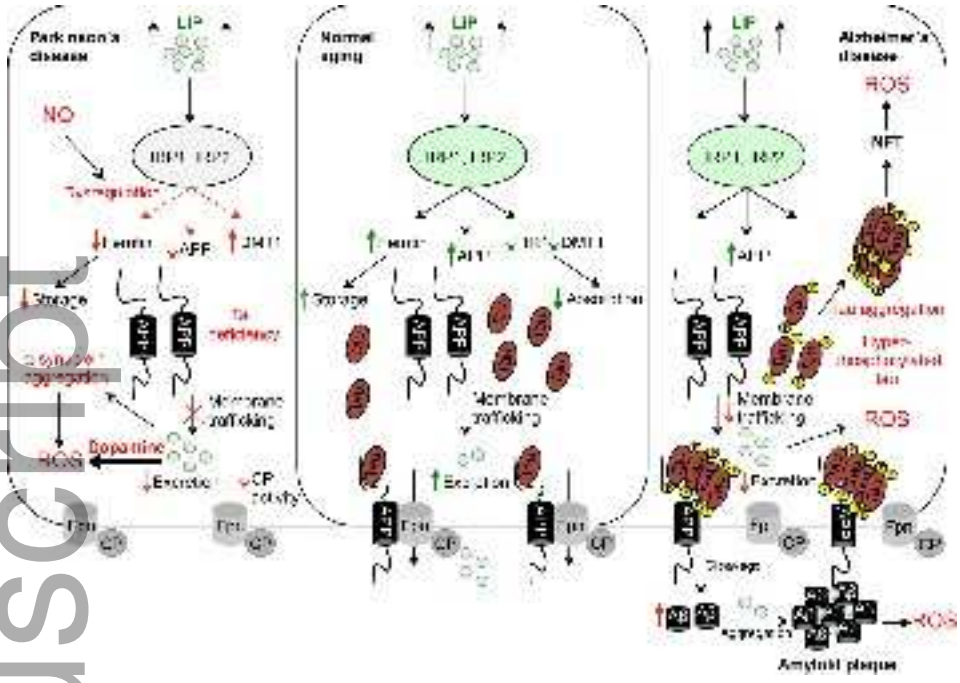

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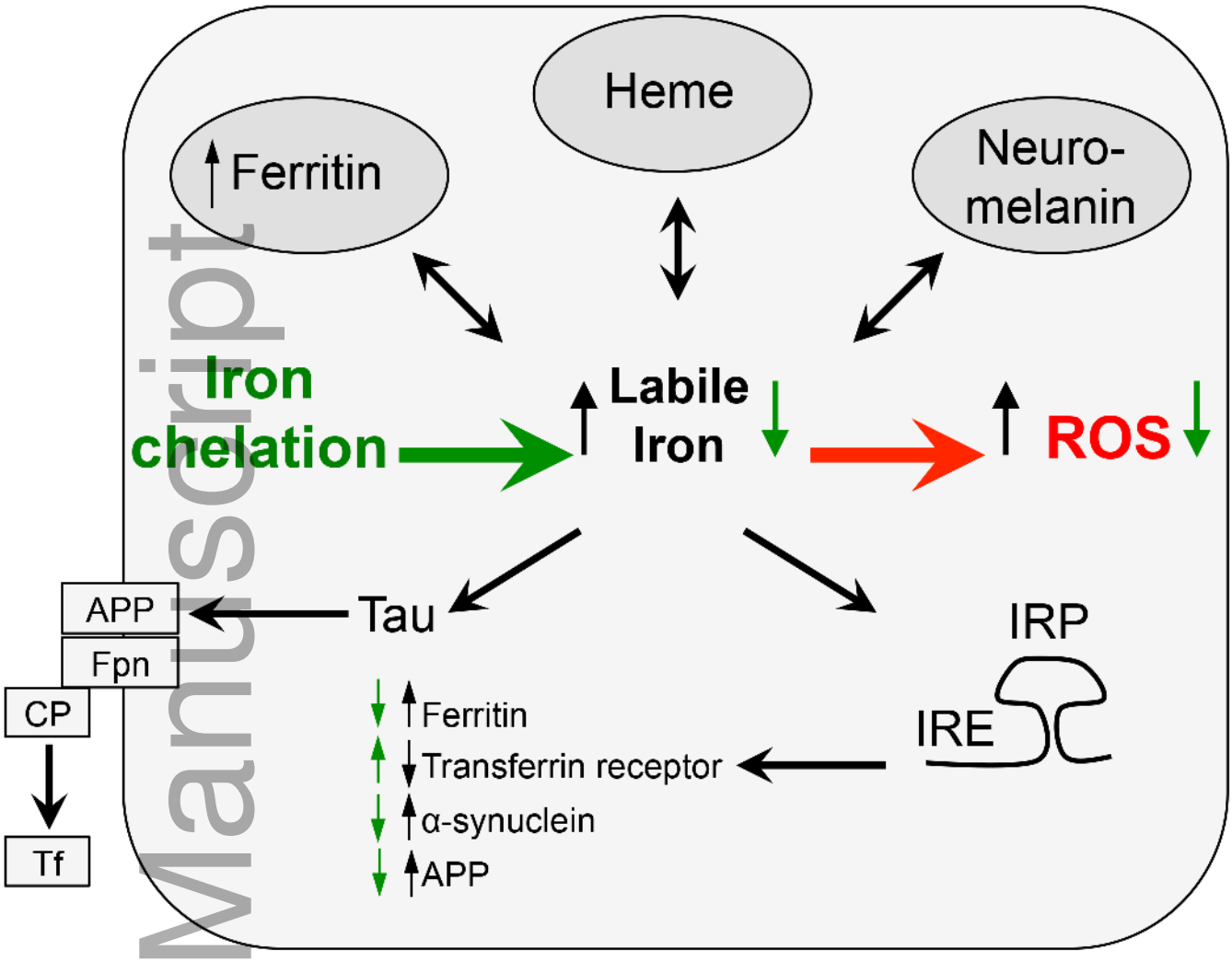

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