Iron Neurotoxicity in Parkinson's Disease

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Abstract

Iron plays essential roles in the early development of cognitive processes and in the maintenance of neuronal functions in the mature brain; therefore, neurons have expeditious mechanisms to ensure a readily available iron supply. However, several

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neurodegenerative diseases present dysregulation of iron homeostasis derived from mitochondrial dysfunction, inflammatory conditions, decreased glutathione levels, and oxidative damage, resulting in downstream protein aggregation, lipid peroxidation, and nucleic acid modification. In this chapter, the mechanisms by which iron homeostasis is lost in Parkinson's disease (PD) are discussed. The relevance of endogenous toxins such as mediators of mitochondrial dysfunction, the relationship between inflammation and iron dyshomeostasis, and the role of hepcidin as a neuroprotective agent are also addressed. A model is proposed that involves a positive feedback loop between mitochondrial dysfunction, inflammation, and increased iron content in dopaminergic neurons, which, if unchecked, ends in substantia nigra (SN) neuronal death.

Keywords

Parkinson's disease · Brain iron · Neurodegeneration · Mitochondrial dysfunction · Hepcidin · Inflammation

Abbreviations

6-OHDA	6-hydroxydopamine
AD	Alzheimer's disease
ALAS2	5′-aminolevulinate synthase 2
APP	Amyloid precursor protein
ARE	Antioxidant response element
ATP13A2	ATPase cation transporting 13A2
Aβ	Amyloid β
CDC14A	Dual specificity protein phosphatase
CDKAL1	CDK5 Regulatory Subunit Associated Protein 1 Like 1
CNS	Central nervous system
CP	Ceruloplasmin
CSF	Cerebrospinal fluid
Dexras1	Dexamethasone-induced Ras protein 1
DJ-1	PARK7, Parkinson disease protein 7
DMT1	SLC11A2, divalent metal transporter 1
Erv1	Mitochondrial FAD-linked sulfhydryl oxidase ERV1
FBXL5	F-Box and Leucine-Rich Repeat Protein 5
Fe-S	Iron-sulfur
FPN1	SCL40A, ferroportin 1
GPX4	Glutathione peroxidase 4
HIF	Hypoxia inducible factor
HRE	HIF response element
IFNγ	Interferon gamma
IL	Interleukin
IRE	Iron responsive element
IRP	Iron regulatory protein
KIF4A	Kinesin Family Member 4A

L-DOPA	L-3,4-dihydroxyphenylalanine
LIMK1	LIM domain kinase 1
LIP	Labile iron pool
LPS	Lipopolysaccharide
LRRK2	Leucine-rich repeat kinase 2
L-VGCC	L-type voltage-gated calcium channel
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRCKα	Myotonic dystrophy kinase-related Cdc42-binding kinase α
NDUFS4	NADH-ubiquinone oxidoreductase subunit S4
NFκB	Nuclear factor kappa B
Nfs1: NMDA	*N*-Methyl-D-aspartate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
Nrf2	Nuclear factor erythroid 2-related factor 2
NTBI	Non-transferring bound iron
$ONOO^-$	Peroxynitrite
PAP7	PKA-associated protein 7
PD	Parkinson's disease
PINK-1	PTEN-induced putative kinase 1
PSP-PDC	Progressive supranuclear palsy, parkinsonism-dementia complex
PUFAs	Polyunsaturated fatty acids
RBC	Red blood cells
ROS	Reactive oxygen species
SN	Substantia nigra
Steap	Six-transmembrane epithelial antigen of the prostate
Tf	Transferrin
TfR1	Transferrin receptor 1
TLR4	Toll-like receptor 4
TNF	Tumor necrosis factor
UPDRS	Unified Parkinson's Disease Rating Scale
UTR	Untranslated region

1 Introduction

Current views depict PD as a multifactorial neurodegenerative condition characterized by the loss of dopaminergic neurons of the SN pars compacta, which results in the appearance of both non-motor and motor pathognomonic signs of the disease. Early observations in postmortem PD tissue showed decreased glutathione content and increased oxidative-mediated damage, establishing the evidence for the participation of oxidative stress in the nigral neuronal death occurring in PD.

An important development in the study of the molecular mechanisms underlying PD arose from the serendipitous discovery of 1-methyl-4-phenyl-1,2,3,6-

tetrahydropyridine (MPTP) as a parkinsonian toxin. MPTP crosses the blood-brain barrier, and it is oxidized by astrocytes to the active metabolite MPP^+. Subsequently, MPP^+ is selectively taken up by dopaminergic neurons through the dopamine transporter DAT. Inside neurons, MPP^+ inhibits mitochondrial complex I, decreasing ATP levels and increasing reactive oxygen species (ROS) production beyond physiological levels, leading to oxidative stress.

Additionally, genetic mutations that induce familiar parkinsonism provided important insights into the cellular and molecular processes that go awry in PD. PINK1, parkin, DJ-1, and α-synuclein are proteins that interact with the mitochondrion and modulate its function. The fact that the loss of function of these proteins results in familiar PD supports the notion that mitochondrial dysfunction is a central event in PD pathogenesis. Why mitochondrial dysfunction occurs and how it interacts with other pathological factors to promote disease progression are most active research areas in the study of PD.

Likewise, inflammation in the central nervous system is a condition strongly associated with neuronal death in several neurodegenerative disorders, including PD. Inflammation is characterized by the occurrence of reactive microglia and astroglia as well as a massive production of pro-inflammatory cytokines.

Here, the present evidence that links mitochondrial dysfunction, iron dyshomeostasis, and inflammation during the process of neuronal degeneration in PD will be discussed.

2 Iron Homeostasis and Dyshomeostasis in Parkinson's Disease

2.1 The Iron Responsive Element – Iron Regulatory Protein (IRE/IRP) System: A Brief Description

In vertebrates, cellular iron levels are posttranscriptionally controlled by the activity of iron regulatory proteins (IRP1 and IRP2), cytoplasmic proteins that bind to structural elements called iron-responsive elements (IREs). IREs are found in the untranslated region of the mRNAs of the major proteins that regulate cellular iron homeostasis. These proteins include the transferrin receptor 1 (TfR1) and the iron transporter DMT1, both involved in iron import, and the iron storage protein ferritin and the iron exporter ferroportin 1 (FPN1), involved in decreasing cytosolic redox-active iron levels (Papanikolaou & Pantopoulos, 2017).

Both IRP1 and IRP2 activities respond to changes in cellular iron status, although through different mechanisms, because IRP1, unlike IRP2, is a bifunctional protein with two mutually exclusive activities. In iron-replete conditions, IRP1 has a 4Fe-4S cluster that renders the protein active as a cytoplasmic aconitase (c-aconitase) but inactive for IRE-binding, thus promoting ferritin and FPN1 mRNA translation; moreover, in iron-replete conditions, TfR1 and DMT1 mRNAs are degraded by nucleases, whereas IRP2 undergoes proteasomal degradation. In contrast, low levels of intracellular iron decrease Fe-S cluster biosynthesis, which causes IRP1 and IRP2

to bind and stabilize TfR1 and DMT1 mRNAs and to suppress ferritin and FPN1 mRNA translation.

Besides being regulated by iron levels, IRP1 is also activated by reactive oxygen/nitrogen species, in particular nitric oxide and peroxynitrite. The conversion of c-aconitase to IRP1 by nitric oxide resembles a two-step mechanism. The first step involves oxidant-mediated cluster disruption and the concomitant formation of a disulfide bridge, which is unable to bind IREs. The activation of its RNA-binding ability requires disulfide bridge breakage by a reducing system like thioredoxin, in a second step. Peroxynitrite, produced by the reaction of nitric oxide with the superoxide anion, shows a biphasic behavior. At high concentrations, it causes tyrosine nitration of IRP1, inhibiting both its aconitase and IRE-binding activities, whereas at low concentration, peroxynitrite induces Fe-S cluster disassembly, promoting IRP1 IRE-binding activity.

IRP1 activation by H_2O_2 and requires membrane-associated components, pointing to an indirect signaling-triggered event. Since H_2O_2 quickly activates both the endothelial and neuronal nitric oxide synthases eNOS and nNOS (Kellogg et al., 2017), it is arguable that IRP1 activation by H_2O_2 could be mediated by nitric oxide production.

In contrast to IRP1, IRP2 activity is downregulated through proteasomal degradation mediated by the ubiquitin ligase FBXL5. FBXL5 is a singular triple detector of iron availability, oxygen levels, and [Fe-S] clusters biosynthesis. The N-terminal domain of FBXL5 folds in a hemerythrin fold, with a diferric center. Under iron depletion or hypoxic conditions, the FBXL5 N-terminal domain partially unfolds, allowing for its polyubiquitination followed by FBXL5 proteasomal degradation (Moroishi et al., 2014). On the other hand, under oxidative stress conditions when iron levels are high, FBXL5 is folded in a compact structure resistant to degradation. FBXL5 has a redox-active [2Fe-2S] cluster in its C-terminal domain. The oxidized state of the Fe-S cluster is essential for IRP2 recognition. In this way, IRP2 accumulates under low iron and/or hypoxic conditions. In agreement with the relevance of FBXL5-IRP2 axis in physiological control of iron homeostasis, FBXL5 KO mice present embryonic lethality, which is prevented by the additional deletion of the IRP2 gene (Moroishi et al., 2014).

The genetic ablation of IRP2 revealed that $IRP2^{-/-}$ mice are born normal but develop in adulthood a movement disorder characterized by ataxia, bradykinesia, and tremor. In contrast, $IRP1^{-/-}$ mice are apparently normal during adulthood, with only slight misregulation of iron metabolism in the kidney and brown fat tissue, and develop polycythemia. Moreover, the double knockout mice for IRP1 and IRP2 die before birth, indicating that IRE/IRP system plays a crucial role in maintaining iron homeostasis, where IRP2 seems to dominate the physiological regulation of iron metabolism whereas IRP1 seems to predominate in pathophysiological conditions.

A genome-wide search for IRE sequences resulted in 35 new target genes for IRPs (Sanchez et al., 2011). This data indicates that the scope of the IRE/IRP system has lately gone from a restricted iron homeostasis-related protein to a more global system that links iron homeostasis to a myriad of other cellular functions (Fig. 1).

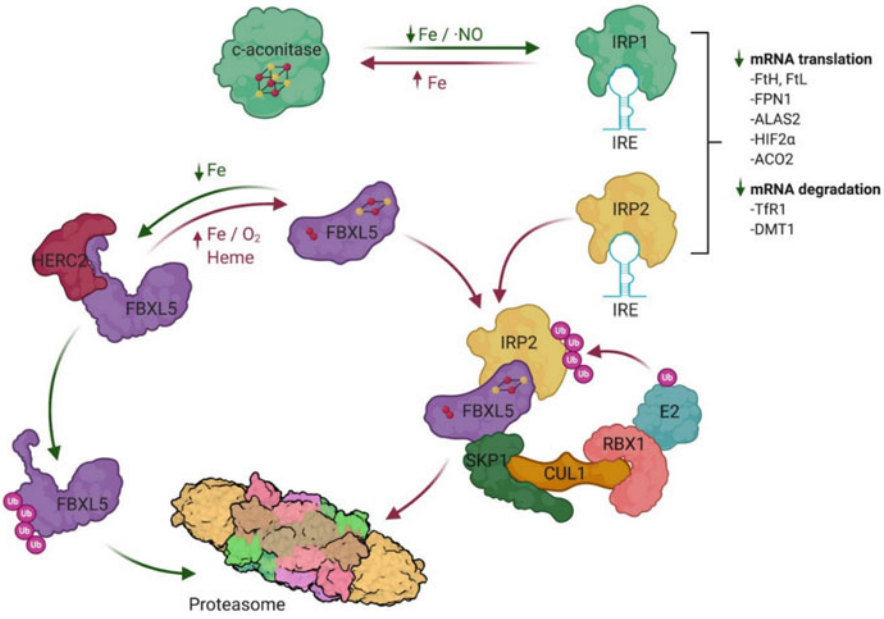

Fig. 1 A scheme of IRP1 and IRP2 regulation. IRP1 is regulated by intracellular iron levels and the presence of nitric oxide (·NO), whereas IRP2 activity is downregulated through proteasomal degradation mediated by the ubiquitin ligase FBXL5, a singular triple detector of iron availability, oxygen levels, and [Fe-S] cluster biosynthesis (for details, please see text)

Genes containing IRE motif include HIF-2α, CDC14, MRCKα, ALAS2, and profilin. HIF-2α regulates the expression of proteins involved in erythropoiesis, angiogenesis, programmed cell death, cancer, and ischemia. CDC14, a tyrosine phosphatase involved in mitotic exit and initiation of DNA replication, has been shown to dephosphorylate the tumor suppressor protein p53, possibly regulating its function. MRCKα, a serine/threonine kinase regulated by Cdc42, is a member of the Rho subfamily of small GTPases that regulate cytoskeletal organization. MRCKα also regulates myosin assembly by phosphorylation of the myosin binding subunit of myosin light chain and phosphorylates LIMK1 and LIMK2, protein kinases that regulate actin assembly.

Several findings strongly suggest that iron accumulation-induced dysfunction of the IRE/IRP system can contribute to protein aggregation and neuronal loss in neurodegenerative diseases like AD and PD. There is a functional IRE motif in the 5′-UTR mRNA of the amyloid precursor protein (APP) (Rogers et al., 2002). An increase in iron levels enhances APP mRNA translation and amyloid β (Aβ) generation. Moreover, iron chelators selectively downregulate the translation of APP mRNA. Interestingly the use of chemical inhibitors of IRE in APP mRNA reduces APP and amyloid levels as well as cognitive decline in the TgCRND8 AD mouse model (Tucker et al., 2006). The α-synuclein mRNA also has an IRE-like motif located in the 5′ UTR (Friedlich et al., 2007).

2.2 Molecular Components of Neuronal Iron Homeostasis

The molecular components of neuronal iron homeostasis are shown in Fig. 2.

The scheme includes transferrin (Tf) and TfR1, inflow (DMT1) and efflux (FPN1) iron transporters, the iron storage protein ferritin, the ferrireductase Steap2 that reduces Fe^{3+} to Fe^{2+} prior to transport by DMT1, and the ferroxidases hephaestin or ceruloplasmin responsible for the oxidation of Fe^{2+} after transport by FPN1 and prior to the binding by apoTf. Tf-bound iron uptake starts with the binding of transferrin to surface receptors, followed by internalization into the endosomal system, release of iron mediated by the acidification of the endosome, reduction by Steap2, and transport into the cytoplasm by endosomal DMT1. Once in the cytoplasm, Fe^{2+} becomes part of the labile or reactive iron pool where it distributes to mitochondria, neuromelanin, and ferritin or it engages in electron exchange reactions. All the components described in Fig. 1 are expressed in the brain.

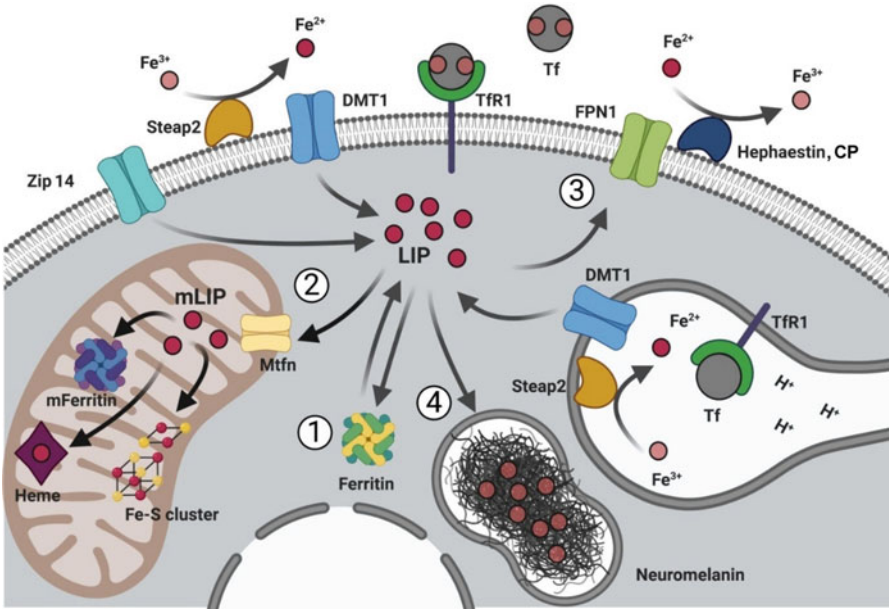

Fig. 2 Molecular components of neural iron homeostasis. The molecular components comprise the transferrin-transferrin receptor complex, the ferrireductase Steap2, responsible for the reduction of Fe^{3+} prior to transport by DMT1, iron transporters (DMT1, Zip14, Mitoferrin2 (Mtfn), and Ferroportin1 (FPN1)); the iron storage protein ferritin, the ferrireductase Steap2, responsible for the reduction of Fe^{3+} prior to transport by DMT1 in endosomes in the brain; and the ferroxidases hephaestin and CP, responsible for the oxidation of Fe^{2+} after transport by FPN1 and prior to Fe^{3+} binding to apoTf. Once in the cytoplasm, iron forms part of the cytoplasmic labile iron pool (cLIP) from where it distributes to mitochondria, ferritin, and neuromelanin, or it is exo-transported by FPN1. In the cellular environment, cLIP iron is a net ROS producer. For simplicity, the molecular components of mitochondrial iron homeostasis were omitted

2.2.1 Iron Uptake Systems

The iron concentration in cerebrospinal fluid (CSF) ranges between 0.2 and 1.1μM, whereas the Tf concentration lies around 0.24μM (Symons & Gutteridge, 1998). Thus, CSF iron often exceeds the binding capacity of Tf, and brain cells may undergo both Tf-bound and non-Tf-bound (NTBI) iron uptake. The last one is expected to occur in neurons that express DMT1 or other iron uptake transporters.

DMT1 (SLC11A2) is the paradigmatic transporter that delivers iron inside cells. In the brain, DMT1 is expressed in hippocampal pyramidal and granule cells, cerebellar granule cells, pyramidal cells of the piriform cortex, SN, and the ventral portion of the anterior olfactory nucleus, striatum, cerebellum, hippocampus, and thalamus, as well as in vascular cells throughout the brain and ependymal cells in the third ventricle. The ubiquitous presence of DMT1 in neurons suggests that DMT1 is necessary for their fundamental physiological functions.

The mammalian DMT1 gene has alternative transcription start sites and undergoes alternative splicing generating four isoforms, all with similar activities for Fe^{2+} transport. The 1A and 1B mRNA DMT1 variants originate from alternative transcription start sites at the 5′ end (exons 1A and 1B), while the +IRE or –IRE variants originate from alternative splicing on the 3′ end (exons 16/16A and 17). The 1B isoforms are expressed in the brain, whereas the 1A isoforms are almost exclusively expressed in the intestine (Hubert & Hentze, 2002).

Given its crucial role on cell iron uptake, knowledge on the regulation of DMT1 expression is essential to understand pathological conditions in which iron accumulation is observed. It is generally accepted that the +IRE isoforms of DMT1 are regulated by the IRE/IRP system, which posttranscriptionally regulates the expression of iron homeostasis proteins such as TfR1, DMT1, FPN1, and ferritin, in response to the concentration of reactive iron in the cytoplasm.

Knowledge on the regulation of DMT1(-IRE) mRNA levels through its 3'UTR region is scarce. A recent study reported its regulation in developing erythroid cells by the miRNA Let-7d (Andolfo et al., 2010). The authors demonstrated that the expression of DMT1(-IRE) decreases when the levels of miRNA Let-7d increase, probably by inducing its degradation through binding to imperfect complementary sites within the 3'-UTR of its mRNA. The expression of DMT1 is repressed also by the microRNA-16 family members miR-16, miR-195, miR-497, and miR-15b (Jiang et al., 2019).

DMT1 levels are also regulated at the posttranslational level. α-Synuclein induces phosphorylation of Parkin at Ser131 which, by reducing DMT1 ubiquitination, increases iron influx and oxidative stress (Bi et al., 2020). Neuroinflammation also upregulates DMT1 activity by NO-mediated S-nitrosylation. Of note, DMT1-overexpressing mice show a normal motor phenotype despite a selective increase of iron levels in the SN pars compacta. A compensatory upregulation of Parkin, which ubiquitinates lactotransferrin, reducing intracellular iron levels, could explain this unanticipated result.

Iron also enters neuron through L-type calcium channels (L-VGCC) since MPTP/MPP^+-induced DA neuron death is inhibited by L-VGCC blockers like nicardipine, nifedipine, and isradipine (reviewed in (Núñez & Hidalgo, 2019). Other studies have

provided evidence of iron influx through NMDA receptors in hippocampal neurons. The question remains as to whether the protective effect of L-VGCCs blockers is due to decreased iron and/or decreased calcium influx. Since iron-mediated oxidative stress results in increased intracellular calcium (Núñez & Hidalgo, 2019), iron entrance through L-VGCCs should induce increased calcium content, thus aggravating iron-induced neurotoxicity.

2.2.2 The Iron Exporter FPN1

FPN1 is the only member of the SLC40 family of transporters and the first reported protein that mediates iron efflux from cells. This protein is expressed in numerous cell types, including two types that are important in systemic iron homeostasis, enterocytes, and macrophages. In enterocytes, FPN1 is responsible for iron efflux during intestinal iron absorption, while in Kupffer cells, FPN1 mediates iron export for reutilization by the bone marrow.

FPN1 expression is regulated at several levels, including transcriptional and translational mechanisms. The FPN1 promoter contains an antioxidant response element, which can be repressed or activated by transcription factors Bach1 and Nrf2. In addition, the FPN1 promoter also contains HIF response elements targeted by HIF2α, inducing upregulation of iron exporter expression.

At the translational level, FPN1 is regulated by the IRE/IRP system. There are two closely related isoforms of FPN1, one (FPN1A) that has an IRE motif in the 5'-UTR and another (FPN1B) that does not have it and is generated from an alternative upstream promoter (Zhang et al., 2009). The 5'-UTR motif gives FPN1 a ferritin-like response to variations in cell iron content, increasing its expression under elevated cell iron conditions and decreasing its expression on iron deprivation. On the contrary, the FPN 1B isoform is only expressed in enterocytes and erythroid precursor cells, which under iron limitation conditions is continually translated allowing iron supply to other tissues that require it (Zhang et al., 2009). The posttranslational regulation of FPN1 expression is mediated by hepcidin (see below).

2.2.3 TfR1

TfR1 mediates the incorporation of iron into cells through clathrin-mediated endocytosis of the TfR1-Tf-Fe complex, with recycling of TfR-apoTf. The presence of TfR1 in neurons, microglia, and astrocytes and its response to iron deficiency have been described. Early studies by Faucheux et al. revealed that the density of 125I-labeled transferrin binding sites was highest in the central gray substance, intermediate in the catecholaminergic cells of the superior colliculus and ventral tegmental area, and almost nonexistent in the SN. Interestingly, during aging the TfR1 expression in the hippocampus, striatum, and SN is reduced, but in the cortex, the TfR1 protein levels remain unchanged. The decreased expression of TfR1 during aging suggests a compensatory mechanism to decrease iron uptake in aged cells.

As noted above, the TfR1 is posttranscriptionally regulated by the IRE/IRP system. When cellular iron is low, IRPs bind to five IRE motifs in the 3'-UTR of TfR1 mRNA increasing its stability. TfR1 expression is also transcriptionally regulated by hypoxia through HIF-1 and by inflammatory stimuli: LPS/IFNγ treatment to

mouse macrophages upregulates NF-κB, which in turn transiently activates HIF-1-dependent TfR1expression and iron uptake.

2.2.4 Ferritin

Ferritin, a multimeric protein formed by 24 subunits of H and L monomers, is the only well-characterized iron storage protein in living organisms. H- and L-ferritin form a hollow cavity that can store as much as 4500 Fe^{3+} atoms in crystallized diferric oxo-hydroxyl complexes. Ferritin plays a fundamental role in controlling the size of the cytoplasmic redox-active iron pool. Although ferritin is expected to reduce this pool by storing iron, there is a dynamic exchange of iron between its ferritin-bound form and a cytoplasmic form amenable for transport by FPN1. In addition, ferritin contributes to the pool of redox-active iron every time it undergoes lysosomal degradation. A lack of upregulation of ferritin by increased iron content was observed in PD patients (Faucheux et al., 2002). This lack of regulation may be due to the increased IRP1 activity observed in experimental rodent models of PD (Salazar et al., 2006).

2.2.5 The Ferrireductases and Ferroxidases

Fe^{2+} is the preferred species for transport, so ferrireductases must reduce Fe^{3+} prior to its transport into the cell. In contrast, Fe^{2+} transported from inside the cell to the extracellular milieu must be oxidized by ferroxidases to Fe^{3+} prior to transferrin binding.

The Steap family of ferrireductases comprises the members Steap1, Steap2, Steap3, and Steap4 (Ohgami et al., 2006). Steap3 was described in endosomes of erythroid precursor cells, catalyzing the reduction of Fe^{3+} to Fe^{2+} prior to transport by DMT1. In situ hybridization studies show that Steap2 is expressed in the brain (Ohgami et al., 2006). As described above, Steap2 has a role in iron uptake by reducing Fe^{3+} to $Fe2^{+}$ both at the plasma member and endosomes.

Two multi-copper ferroxidases, ceruloplasmin (CP) and hephaestin, are involved in cellular iron export. CP seems to be required for iron release from most tissues in the body. Although the liver is the predominant source of serum CP, CP gene expression has also been demonstrated in many tissues including the spleen, lung, testis, and brain. CP is expressed in astrocyte membranes as a GPI-anchored protein. Apparently, this isoform is the main entity responsible for iron efflux from astrocytes since the soluble CP in CSF is insufficient. Hephaestin in mainly expressed in oligodendrocytes. Since CP is mainly expressed in astrocytes and hephaestin in oligodendrocytes, the absence of these proteins induces iron accumulation and oxidative stress in a cell type-specific manner (Chen et al., 2019).

Reduced serum ceruloplasmin level exacerbates nigral iron deposition in PD, whereas patients with aceruloplasminemia (CP absence or dysfunction) develop Parkinson-like symptoms of tremors and other muscle movement issues. During the aging process, the protein levels of CP significantly increase in striatum and SN, suggesting an important role for CP in the regulation of cellular iron in these tissues. Interestingly, in the cortex and hippocampus, the protein levels of CP remain

unchanged with age (Lu et al., 2017), an observation that has not been further explored in its functional consequences.

Alpha-synuclein is a metal-binding protein with ferrireductase activity that can reduce Fe^{3+} using copper as electron donor. The observation that α-synuclein overexpression results in the increased free radical production led to the proposal that if unchecked, the ferrireductase activity of α-synuclein may cause iron dyshomeostasis followed by increased ROS production, increased intracellular calcium, and apoptotic cell death (Sian-Hulsmann & Riederer, 2020).

2.2.6 Neuromelanin

Neuromelanin is a dark aggregate that accumulates both in the SN and locus coeruleus monoaminergic neurons. Neuromelanin is formed by oxidized metabolites of dopamine, containing a peptide component of about 15%. In SN neurons, neuromelanin accumulates progressively with age until occupying most of the neuronal cytoplasm (Zecca et al., 2001).

Neuromelanin avidly binds Fe^{3+}, both in high and low affinity binding sites. Given the low expression of ferritin, neuromelanin is the main iron storage moiety in SN pars compacta neurons (Zecca et al., 2001). It is believed that the high affinity sites are protective as they sequester iron in a redox-inactive form, whereas iron in the low affinity sites is redox-active (Gerlach et al., 2008). Thus, neuromelanin can have both neuroprotective and neurotoxic roles in dopaminergic neurons. Its strong iron-chelating properties diminish redox-active iron, but if its high affinity iron binding capacity is surpassed, for example, by increased iron content during aging, low affinity neuromelanin-bound iron becomes a source of the highly toxic hydroxyl free radical, and the neurons, in turn, become prone to oxidative damage (Xing et al., 2018).

2.3 Iron Accumulation and Iron Chelation Therapy in PD

Iron is an intrinsic ROS producer. When one or more of its six coordination sites is not tightly bound, iron engages in one-electron exchange reactions, producing free radicals through the Haber-Weiss and Fenton reactions. The thermodynamic balance of these reactions indicates that in the reductive environment of the cell, iron, in the presence of oxygen, catalyzes the consumption of GSH and the production of the hydroxyl radical (Núñez et al., 2012). In dopaminergic cells, another source of free radicals derives from the enzymatic and nonenzymatic oxidation of dopamine mediated by redox-active iron, resulting in semiquinones and H_2O_2 production. Thus, redox-active iron, both through the Fenton reaction or via dopamine oxidation, is a dangerous prooxidant agent.

Overwhelming evidence indicates that iron accumulation is a common feature of a number of neurodegenerative disorders of the CNS that include Huntington's disease, Alzheimer' disease, Friedreich's ataxia, amyotrophic lateral sclerosis, and PD. Furthermore, in PD patients, a direct association was found between brain iron content and the decline of movement and cognitive parameters like the Unified Parkinson's Disease Rating Scale, rapid eye movement during sleep, sense of smell, and depression. These findings suggest that elevated iron content in SN neurons is

associated with the neurodegenerative process. Indeed, iron accumulation has been directly demonstrated in the dopaminergic neurons of the SN.

The contribution of iron dyshomeostasis to the progression of events leading to neuronal death is likely, since iron accumulation has been detected in brain tissue from patients who have died after the final steps of the pathology. Since neuronal death caused by MPTP or 6-hydroxydopamine intoxication is blocked by pharmacologic or genetic chelation of iron, it is possible that iron dyshomeostasis takes place in the late stages of the disease as part of a vicious cycle resulting in uncontrolled oxidative damage and cell death. A study in mesencephalic dopaminergic neurons shows that low (0.25–0.5μM) concentrations of MPP^+ induce neuritic tree collapse without loss of cell viability (Gomez et al., 2011). This collapse was effectively prevented by antioxidants and by iron chelators. Thus, increased intracellular iron and ROS most probably are involved in the early steps of dopaminergic neuron dysfunction, prior to cell death. Later, a vicious cycle of further iron accumulation, complex I dysfunction and ROS increase may result in uncontrolled oxidative damage and cell death.

Iron toxicity is not restricted to dopaminergic neurons. Neurotoxic concentrations of NMDA induce the NO-Dexras1-PAP7 signaling cascade in glutamatergic PC12 cells. Upon activation, PAP7 binds to intracellular DMT1 and relocates it to the plasma membrane, increasing intracellular iron and the production of hydroxyl radicals. Thus, the DMT1-iron uptake-hydroxyl radical signaling pathway appears to mediate NMDA neurotoxicity (Cheah et al., 2006).

Lately, iron chelation has been introduced as a new therapeutic concept for the treatment of neurodegenerative diseases with a component of iron accumulation, as detailed in recent reviews (Núñez & Chaná-Cuevas, 2018). Effective chelators for the oral treatment of neurodegenerative diseases must cross both the intestinal barrier and the blood-brain barrier. In two recent randomized placebo-controlled clinical trials, 40 patients in early stages of PD treated with the iron chelator deferiprone at doses of 30 mg/kg body weight showed significant reduction in iron deposits and oxidative damage in the SN, together with improved motor indicators of disease progression. Yet, albeit this treatment was not completely harmless since one case of agranulocytosis and two of neutropenia were reported, the authors concluded that the beneficial results justify a comprehensive assessment of iron chelation therapy for PD. In a recently finished randomized, placebo-controlled trial, 22 early-onset PD patients were administered deferiprone or placebo, for 6 months (Martin-Bastida et al., 2017). Although patients treated daily with 30 mg/kg deferiprone showed a trend for improvement in the Unified Parkinson's Disease Rating Scale (UPDRS) scores and quality of life, the differences did not reach significance. The authors again concluded that the results support more extensive clinical trials into the potential benefits of iron chelation in PD (Martin-Bastida et al., 2017). An extended, phase II clinical trial is currently underway (https://clinicaltrials.gov/ct2/show/NCT02655315) by a conglomerate of 21 clinical centers in 8 European countries (Moreau et al., 2018). This massive endeavor strongly supports the feasibility of iron chelation as a viable therapeutic approach for the treatment of PD.

3 Fe-S Clusters and PD

Iron-sulfur (Fe-S) clusters are small inorganic cofactors formed by tetrahedral coordination of iron atoms with sulfur groups. The Fe-S clusters most found in eukaryotes are [2Fe–2S] and [4Fe–4S]. Fe-S clusters are involved in many cellular processes, including electron transport, enzymatic catalysis, and DNA synthesis and repair.

The mitochondrion plays a central role in the generation and biology of Fe-S clusters since it holds the assembly machinery responsible for their synthesis. Fe-S cluster synthesis occurs in the mitochondrion and the cytoplasm. In eukaryotes, the Fe-S cluster assembly machinery of mitochondria comprises the cysteine desulfurase Nfs1, which provides sulfur, and Isu1, a protein that serves as a molecular scaffold for the assembly of the Fe-S cluster. Synthesis of the transiently bound Fe-S cluster on the Isu scaffold proteins requires Fe^{2+} and an electron donor, presumably to generate sulfide (S^{2-}) from cysteine. Finally, synthesized Fe-S clusters are either transferred to mitochondrial apoproteins or are exported into the cytoplasm by the Fe-S cluster export machinery. This machinery involves the ABC-B7 transporter, the antioxidant GSH, and the sulfhydryl oxidase Erv1, which is also involved in protein import.

Fe-S cluster proteins fulfill numerous functions in eukaryotic cells. Mitochondrial Fe-S proteins take part in the electron transport chain (complexes I–III), lipid β-oxidation, heme biosynthesis, and the citric acid cycle. Cytosolic and nuclear Fe-S proteins participate in iron homeostasis, tRNA modification, translation, nucleotide metabolism, mitosis, and DNA synthesis and maintenance. An updated compendium of eukaryotic Fe-S cluster-containing proteins is provided by a recent review (Lill & Freibert, 2020). Prominent members of the ever-expanding Fe-S cluster protein family include CDKAL1, an enzyme that catalyzes methylthiolation of $tRNA^{Lys}_{UUU}$, regulating insulin translation and secretion; the chromokinesin KIF4A, a molecular motor associated to chromosome segregation during mitosis; the primase, an enzyme essential for RNA primer synthesis; and the catalytic subunit of DNA polymerase ε, the major DNA polymerase of leading strand. For a compendium of metalloproteins containing Fe-S clusters, see MetalPDB at http://metalweb.cerm.unifi.it/.

Inhibition of complex I by rotenone results in decreased synthesis of Fe-S clusters, as shown by the decreased activity of the Fe-S cluster-containing enzymes c-aconitase, mitochondrial aconitase, xanthine oxidase, and glycerol-3-phosphate acyltransferase as well as the activation of cytoplasmic IRP1 (Mena, 2011).

Complex I inhibition by rotenone in SH-SY5Y dopaminergic neuroblastoma cells also results in increased IRP1 activity, accompanied by increased levels of TfR1 and DMT1, and decreased levels of FPN1, together with increased iron uptake activity and increased cytoplasmic labile iron pool. IRP1 knockdown abolishes the rotenone-induced increase in iron uptake, and it protects cells from death induced by complex I inhibition. IRP1 knockdown cells present higher ferritin levels, a lower iron labile pool, increased resistance to cysteine oxidation, and decreased oxidative modifications (Urrutia et al., 2017). These results support the concept that mitochondrial dysfunction results in IRP1 activation, triggering iron accumulation and cell death.

Overall, these results are consistent with the view that decreased activity of complex I results, via decreased Fe-S cluster synthesis and the consequent activation

of IRP1, in a false "low iron" signal that activates the iron uptake system. In consequence, diminished Fe-S cluster synthesis could play a fundamental role in the accumulation of iron observed in PD.

4 A Role for Ferroptosis in the Execution Step of Dopaminergic Neuronal Death

Ferroptosis is a newly recognized form of regulated cell death that is characterized by lethal lipid peroxidation downstream of iron accumulation and GSH exhaustion (Stockwell et al., 2017). Ferroptosis vulnerability evolves from polyunsaturated fatty acid incorporation into cellular membranes. The phospholipid peroxidase GPX4 and its cofactor GSH constitute the principal cellular defense against ferroptosis, by converting the potentially toxic lipid hydroperoxides to nontoxic lipid alcohols. Furthermore, dopamine quinone covalently modifies GPX4, reducing its mitochondrial levels (Hauser et al., 2013). Noticeably, ferroptosis is activated earlier than apoptosis in dopaminergic cells treated with low concentration of iron or in mice bearing a pathological α-synuclein mutation (Zhang et al., 2020).

5 Mitochondrial Dysfunction in PD

Mitochondrial dysfunction is another central event involved in PD pathogenesis. Mitochondria not only have a key role in electron transport and oxidative phosphorylation but also are the main cellular source of ROS; in addition, mitochondria are involved in calcium homeostasis and in the regulation and initiation of cell death pathways. Evidence of mitochondrial dysfunction in PD began in the 1980s, when, after an intravenous injection of illicit drugs, four people developed marked parkinsonism. Further analysis of the substances injected revealed the existence of MPTP, which decreases the activity of mitochondrial complex I, as found in postmortem tissue of PD patients, a finding that is probably a founding event in PD-related neuronal death. The association between complex I inhibition and PD is further supported by the observation that rats intoxicated with rotenone, a selective inhibitor of complex I, develop a syndrome similar to PD, characterized by neuronal degeneration and the formation of inclusion bodies rich in α-synuclein. Likewise, inhibition of glutaredoxin 2, an enzyme involved in mitochondrial Fe-S synthesis, produces an alteration in iron metabolism in a model of PD. Additionally, mutations in α-synuclein, Parkin, PINK1, DJ-1, LRRK2, and ATP13A2, disrupt mitochondrial function, resulting in a genetic form of PD; these findings lend further support for an important role of mitochondria in PD neurodegeneration (Smolders & Van Broeckhoven, 2020).

A genetic model of mitochondrial complex I dysfunction through conditional deletion of NDUFS4, an accessory subunit involved in complex I assembly and stability, has been described. NDUFS4 deletion in neurons, astrocytes, and oligodendrocytes causes reactive gliosis in the brainstem and produces motor symptoms such as ataxia (McElroy et al., 2020). In addition, conditional deletion of NDUFS4 specific to dopaminergic neurons in mice does not generate neuronal loss or motor defects but causes, however, a

decline in dopamine levels in the striatum and amygdala and non-motor manifestations, as anxiety and cognitive defects, resembling a presymptomatic stage of PD.

Mitochondrial complex I is essential for NAD^+ regeneration and ATP production. Dissecting both roles in neurodegenerative diseases is fundamental to explain its relevance in pathological mechanisms. The expression of a NAD^+ regenerative enzyme without proton pumping ability (required for ATP synthesis) does not improve $NDUFS4^{-/-}$ mice motor function but ameliorates neuroinflammation and extends lifespan (McElroy et al., 2020), indicating that both phenomena could be dissociated. The authors speculate that the inhibition of the mitochondrial complex I has differential effects on dopaminergic neurons and astrocytes. In neurons, it generates a bioenergetic deficit that has a slight impact on dopamine production/secretion, generating the early non-motor symptoms of PD. On the other hand, in astrocytes, it generates profound metabolic changes, which trigger neuroinflammation and subsequent late neuronal death.

ROS seem to have a negative effect on complex I activity. Experiments with isolated synaptosomal mitochondria revealed that low concentrations of H_2O_2 decrease complex I activity by 10%. This relatively minor effect of H_2O_2 was additive to partial inhibition of complex I induced by low concentrations (5 nM–1μM) of rotenone. Similarly, submitochondrial particles exposed to $O_2^{\bullet-}$, H_2O_2, or $^{\bullet}OH$ present decreased activity of NADH dehydrogenase, a marker of complex I activity. Thus, an initial inhibition of complex I could generate a positive loop between ROS generation and further complex I inhibition.

6 Inflammation, Hepcidin, and PD

In addition to iron accumulation, another event strongly associated with neuronal death in PD and other neurodegenerative disorders is the presence of inflammatory processes characterized by the occurrence of reactive microglia and massive production of pro-inflammatory cytokines. Both phenomena have been studied as independent events leading to the progression of the disease, but, as discussed above, the expression of iron homeostasis proteins such as ferritin and DMT1 is regulated by inflammatory cytokines. Moreover, the identification of hepcidin in the central nervous system reinforces the idea that a chronic inflammation state and deregulation of iron homeostasis are intimately related in PD.

6.1 Hepcidin: The Master Regulator of Iron Homeostasis

Hepcidin is a 25-amino-acid cationic peptide secreted into the bloodstream mainly by the liver. Initially, hepcidin was described as a peptide with antimicrobial activity; however, further studies revealed that it also acts as a major regulator of circulating iron levels (Nicolas et al., 2001).

Two processes contribute to blood iron levels: the recycling of senescent red blood cells (RBC) and intestinal iron absorption. The recycling by spleen macrophages of heme iron from senescent RBC is the main contributor to circulating iron

levels, providing about 95% of daily turnover. The recycling of RBC iron comprises the phagocytosis of senescent RBC, the release of iron contained in the heme moiety of hemoglobin by heme oxygenase-1, and the subsequent iron release into the blood mediated by FPN1. The physiological function of hepcidin is to reduce the levels of circulating iron by downregulation of the iron exporter FPN1 in macrophages. The binding of hepcidin to FPN1 present in the plasma membrane of splenic macrophages induces the endocytosis of the complex and the subsequent lysosomal degradation of FPN1. The decreased levels of FPN1 lead to the accumulation of iron in macrophages and to the decrease of circulating iron.

Hepcidin synthesis is regulated by multiple stimuli that have an effect in the regulation of circulating iron levels. Erythropoietin, a hormone that stimulates red blood cell production, and hypoxia downregulate the synthesis of hepcidin, increasing circulating levels of iron as required for the production of new RBCs. On the contrary, increased plasma iron levels and inflammatory cytokines stimulate hepcidin synthesis, which results in reduced levels of plasma iron and its accumulation in cells. For a recent review, please see (Camaschella et al., 2020).

6.2 Hepcidin Expression in the CNS

Hepcidin shows a wide distribution in the CNS, most notably in the midbrain, with a clear presence in the superior colliculus, the geniculate nucleus, some fiber bundles of the SN pars reticulata, the SN pars compacta, and the striatum.

As stated above, hepcidin synthesis is induced by inflammatory stimuli. Bacterial LPS induces hepcidin expression both in the liver and brain. After an intraventricular injection of LPS, a transient transcription of the gene for hepcidin ensues in the choroid plexus, which correlates with increased levels of pro-hepcidin in the cerebrospinal fluid. The highest hepcidin expression was observed at 3 h, returning to baseline levels 24 h after the injection. Interestingly, LPS treatment induced a tenfold increase in hepcidin expression in the SN, which correlates with a marked increase in iron levels observed in this region in PD. In ischemic brain, there is an increase in hepcidin expression in the cerebral cortex, hippocampus, and corpus striatum, in parallel with increased free iron and ferritin levels.

In a comparative study using primary cell cultures obtained from rat brain, it was observed that inflammatory stimuli induce the expression of hepcidin in astrocytes and microglia, but not in neurons (Urrutia et al., 2013).

6.3 FPN1-Hepcidin Interactions in the CNS

As described above, the hepcidin receptor is the iron exporter FPN1. Interestingly, the spatial distribution of FPN1 and hepcidin are similar, although the effects of hepcidin on FPN1 levels differ according to cell type. The injection of hepcidin in mice lateral cerebral ventricle causes a decrease in the levels of FPN1 in the cerebral cortex, hippocampus, and striatum (Wang et al., 2010). These results prompt the conclusion

that hepcidin generates the same response in the brain as that observed in macrophages, that is, iron retention inside the cells. This conclusion is strengthened by the fact that high doses of hepcidin produce an increase in the iron storage protein ferritin, thus indicating increased cellular iron concentration in these brain areas. Unexpected for a high cell iron situation, treatment with hepcidin induces the decrease of FPN1 protein and mRNA and an increase in total DMT1 in rat hippocampus and cortex (You et al., 2017), a situation that should drive further iron accumulation. Accordingly, hepcidin knockdown protects N27 cells from 6-OHDA-induced apoptosis, decreasing intracellular iron content and oxidative stress. Additionally, in a model of intracerebral hemorrhage, hepcidin knockout rodents show significantly lower brain iron content and oxidative stress and perform better in cognitive tests than their normal counterparts. At the cellular level, hepcidin induces a decrease expression of FPN1 in hippocampal neurons, which is reflected in a reduction of the iron released from these cells and increased cellular iron levels (Urrutia et al., 2013). In cortical microglia and astrocytes, hepcidin induces a reduction of FPN1 expression. However, in microglial cells, hepcidin treatment also induces an increase in protein levels of DMT1, which results in increased iron levels (Urrutia et al., 2013). Interestingly, in microvascular endothelial cells, hepcidin significantly inhibits the expression of TfR1, DMT1, and FPN1 and reduces both iron uptake and iron release, suppressing transport of transferrin-bound iron (Tf-Fe) from the cerebral circulation into the brain. Overall, these results suggest that in microvascular endothelial cells, hepcidin protects from iron accumulation.

Recently, the effect of hepcidin in AD and PD animal models were evaluated. Pretreatment with hepcidin reduces the oxidative damage induced by stereotaxic injection of Aβ peptide (Urrutia et al., 2017). Accordingly, hepcidin overexpression in astrocytes of APP/PS1 transgenic mice reduces iron entry into the brain and accumulation in neurons, attenuates inflammation and oxidative stress, ameliorates Aβ deposition, and rescues cognitive deterioration (Xu et al., 2020). Moreover, in iron-overloaded rats, hepcidin reduces iron accumulation and oxidative damage in the SN. Remarkably, hepcidin overexpression promotes α-synuclein clearance through autophagy and reduces mitochondrial dysfunction and iron accumulation, improving motor deficiency in the 6-OHDA and rotenone PD animal models. Therefore, hepcidin has opposing effects: damage and protection. Apparently, the effects of hepcidin depend on action timing; hepcidin pretreatment protects brain cells from iron load, while hepcidin induction during inflammation aggravates iron load (Vela, 2018). The current view is that the neuroprotective effects of hepcidin are mediated in part by the inhibition of FPN1-dependent iron entrance into the brain through the blood-brain barrier. At present, hepcidin has been proposed as a therapeutic target for the treatment of neurodegenerative diseases with an iron accumulation component.

6.4 Hepcidin: A Nexus Between Inflammation and Iron Accumulation in PD

Many cases of PD are accompanied by general inflammation of the brain, with a dramatic proliferation of reactive amoeboid macrophages and microglia in the SN. In

the striatum, macrophage proliferation is accompanied by high expression of pro-inflammatory cytokines such as TNF-α, IL-1β, IFN-γ, and IL-6 by glial cells. The increase of many of these cytokines has also been observed in cerebrospinal fluid and the basal ganglia of patients with PD. The aforementioned characteristics are also observed in several animal models of PD, such as those injected with 6-hydroxydopamine, MPTP, rotenone, or LPS. For a recent review, please see (Urrutia et al., 2021).

As mentioned above, hepcidin expression is induced by inflammatory stimuli, and its principal function is to reduce protein levels of the iron exporter FPN1 by triggering its lysosomal degradation, reducing iron export. Utilizing isolated neurons, astrocytes, and microglia, it was observed that treatment with TNF-α, IL-6, or LPS increases the expression of DMT1 in neurons, astrocytes, and microglia and that incubation with hepcidin decreases the expression of FPN1 in these three cell types. The net result of these changes was increased iron accumulation in neurons and microglia but not in astrocytes (Urrutia et al., 2013). These results suggest that hepcidin produced by astrocytes and microglia after inflammatory stimuli could be an important contributor to iron accumulation in neurons.

In addition to its role in regulating iron homeostasis, hepcidin has an anti-inflammatory function. The binding of hepcidin to FPN1 activates a signal transduction pathway that reduces the response to LPS, decreasing the production and secretion of IL-6 and TNF-α and reducing the mortality associated with high LPS doses. Accordingly, hepcidin pretreatment reduces Aβ-induced IL-6 and TNF-α expression and secretion from astrocytes and microglia and decreases neurotoxicity and the oxidative damage triggered by Aβ conditioned media. Additionally, hepcidin pretreatment also reduces microglial and astrocyte activation and the neuronal oxidative damage produced by Aβ stereotaxic injection (Urrutia et al., 2013). However, the potential contribution of changes in iron homeostasis in the neuroprotective role of hepcidin needs to be clarified.

6.5 Hepcidin-Independent Relationships Between Iron Accumulation and the Inflammatory Response

Inflammatory cytokines modulate the activity of IRP1 and IRP2 through nitric oxide-dependent and nitric oxide-independent mechanisms, thus influencing iron homeostasis. Stimulation of macrophages with IFN-γ, TNF-α, and LPS produces an increase in the IRE-binding activity of IRP1 and IRP2, reducing FPN1 and increasing DMT1 mRNA expression. Interestingly, pretreatment with the anti-inflammatory cytokine IL-10 reverts this response. In addition, DMT1 expression is transcriptionally enhanced by the transcription factor NFκB, whose activation is downstream of many cytokine receptors such as the TNF receptor and the IL-1 receptor. Activation of NFκB by inflammatory stimuli may play a significant role in iron accumulation by dopaminergic neurons of the SN, which express high levels of TNF receptor.

Interestingly, an increase in the nuclear immunoreactivity of NFκB was observed in PD brains or in animal models of this disease, so it is possible that activation of NFκB contributes to iron accumulation in PD. Accordantly, the pro-inflammatory cytokines TNF-α and IL-6 and the TLR4 agonist LPS directly regulate both mRNA and protein levels of DMT1 and induce a transient decrease in FPN1 protein, generating an increment of iron content in neurons and microglia (Thomsen et al., 2015). Moreover, in cultured ventral mesencephalic neurons, IL-1β or TNF-α treatment leads to increased IRP1 levels, generating an increment of DMT1 and TfR1 protein levels together with a decrease in FPN1, triggering iron accumulation in these cells. Interestingly, the effect of IL-1β or TNF-α over iron deregulation can be suppressed using a NO synthase inhibitor, indicating that IRP1 activation is mediated by NO generation (Wang et al., 2013).

7 A Positive Feedback Loop in the Death of SN Dopaminergic Neurons

The authors propose that inhibition of mitochondrial complex I by endogenous and/or exogenous toxins, and inflammatory processes produced by trauma or other causes, results in a vicious cycle of increased oxidative stress, elevated iron levels, and decreased GSH content (Fig. 3).

In this scheme, neuronal death linked to complex I dysfunction is brought about by a positive feedback loop in which complex I inhibition results in decreased Fe-S cluster synthesis, IRP1 activation, increased DMT1 and TfR1 expression, and iron accumulation. Complex I dysfunction and increased cellular iron result in decreased GSH levels. Both increased oxidative stress and low GSH levels further inhibit complex I activity. Inflammatory cytokines also transcriptionally regulate DMT1, FPN1, and Zip14 synthesis and activate IRPs (see text). Central to this scheme is the deregulation of iron homeostasis since iron chelators effectively block cell death and prevent early events in neurodegeneration, such as neuritic tree shortening.

8 Conclusion

Diminished activity of mitochondrial complex I, iron accumulation, oxidative stress, and inflammation are pathognomonic signs of sporadic PD. It is possible that the initiation of any one of these processes will initiate and enhance the others, through the generation of a positive feedback loop that will produce apoptotic or ferroptotic neuronal death. Intervention of this positive loop should result in prolonged life of the affected neurons. The reasons why SN pars compacta neurons are so particularly prone to this deregulation are largely unknown. Nevertheless, because of their extreme dependence to an adequate energy supply, mitochondrial dysfunction may be a founding event in their neurodegenerative process.

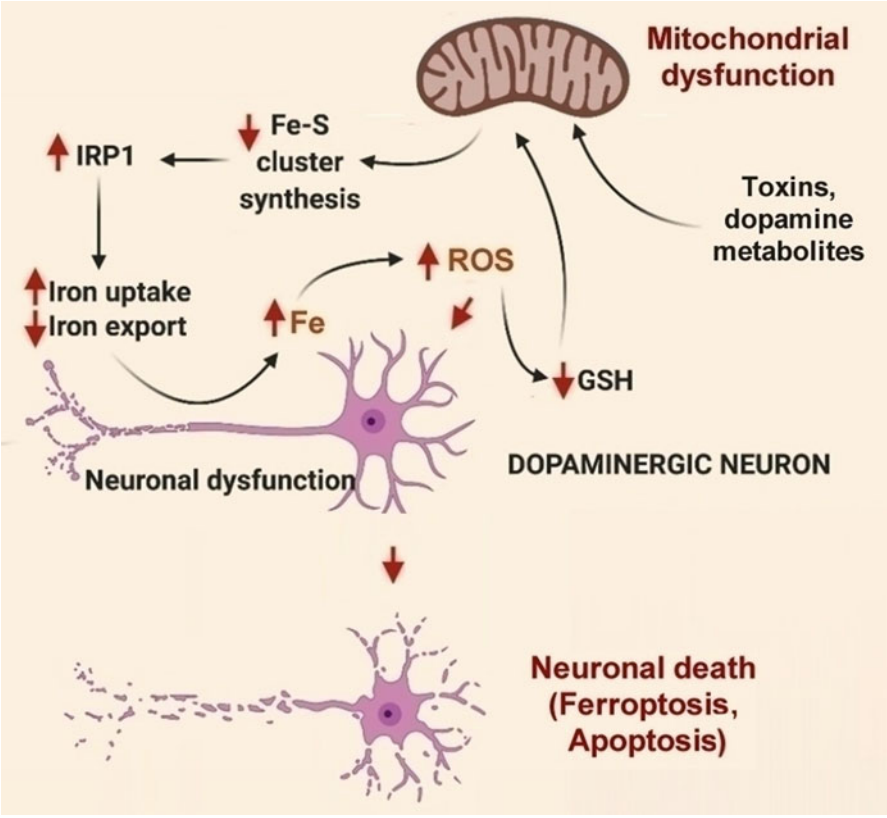

Fig. 3 A positive feedback loop resulting in uncontrolled oxidative load. Complex I inhibition by endogenous or exogenous toxins results in decreased Fe-S cluster synthesis (see text). Decreased Fe-S cluster synthesis leads to the activation of IRP1 that needs a 4Fe-4S cluster to acquire its inactive state. Increased IRP1 activity results in increased DMT1 and decreased FPN1 synthesis, which jointly produce increased iron accumulation. Increased iron induces increased oxidative stress and increased GSH consumption. Decreased GSH produces further complex I inhibition. ROS accumulation induces neuronal damage that ends up in neuronal death by apoptosis or ferroptosis

9 Cross-References

► Iron-Induced Dopaminergic Cell Death In Vivo as a Model of Parkinson's Disease

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