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Endocytic iron trafficking and mitochondria in Parkinson's disease

Silvia Cerri[a,1], Chiara Milanese[b,1], Pier G. Mastroberardino[b,c,*]

[a] Laboratory of Functional Neurochemistry, IRCCS Mondino Foundation, via Mondino 2, 27100, Pavia. Italy
[b] Department of Molecular Genetics, Erasmus University Medical Center Rotterdam, the Netherlands
[c] Department of Life, Health and Environmental Sciences, University of L'Aquila, L'Aquila, Italy

A B S T R A C T

Parkinson's disease (PD) - the second most common neurodegenerative disorder - is a multifactorial disease, the causes of which should be sought in complex and detrimental interactions between genetic and environmental factors. Multiple lines of evidence, however, identify mitochondrial dysfunction, oxidative stress, and iron accumulation as central pathogenic mechanisms. These factors are closely intertwined because mitochondria are a major source of pro-oxidant species and are the major intracellular recipients of iron. How iron is transported to mitochondria, however, is largely unknown. Some studies suggest that trafficking through endocytosis may participate to mitochondrial iron delivery with a "kiss and run" mechanism. Intracellular transferrin levels increase in PD, possibly as a consequence of oxidation of iron-containing prosthetic groups in mitochondria. It is therefore conceivable that transferrin endocytic trafficking can contribute to noxious iron accumulation. This short review will summarize these findings and discuss their relevance for a better understanding of PD pathogenesis.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's and is characterized by a rather selective degeneration of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc). The vast majority of PD cases (> 90%) is sporadic and the causes are likely rooted in the complex interaction between genetic predisposing factor and environmental stimuli. Multiple lines of evidence, however, indicate that oxidative stress and defects in mitochondrial function - and decreased complex I activity in particular - are central to PD pathogenesis (Anandhan et al., 2017). Dopaminergic neurons are characterized by a particularly oxidized intracellular environment also in normal, non-pathological conditions, as indicated by an increased ratio between oxidized and reduced cysteines in glutathione and proteins when compared to neurons in other anatomical regions (Guzman et al., 2010; Mastroberardino et al., 2009, 2008). Consistently, they are particularly vulnerable to toxins perturbing mitochondrial function and leading to increased production of pro-oxidant species, which are typically used to model PD (Martinez and Greenamyre, 2012). Additionally, the parkinsonian SNpc is characterized by markedly increased levels of iron (Berg and Hochstrasser, 2006).

2. Iron

Iron is the most represented transition metal in the cell and is essential for life. Iron can reversibly transition between different oxidation states, most commonly ferrous and ferric iron, i.e. Fe^{2+} and Fe^{3+} respectively. Because ferric iron is scarcely soluble and tends to precipitate, bioavailable iron is principally in the form of Fe^{2+}. Iron is a first raw transition element with incompletely filled *d* orbitals and can therefore easily participate to oxido-reductive (redox) reactions. This property makes this metal particularly suitable as a cofactor in numerous enzymatic reactions, including that catalyzed by tyrosine hydroxylase in the initial and rate limiting step of dopamine synthesis (Nagatsu et al., 2018). The very same redox unique properties, however, render iron also potentially harmful. For instance, ferrous iron very rapidly reacts with hydrogen peroxide to ultimately generate the highly toxic hydroxyl radical (Fenton reaction). In the organism, iron is therefore typically complexed with proteins that serve for its acquisition, transport (e.g. transferrin), or storage means (e.g. ferritin). Details on iron metabolism are summarized in several excellent reviews, e.g. (Chen and Paw, 2012; Pantopoulos et al., 2012). However, for the purposes of this article, it is important to clarify that the vast majority of iron is imported in the cell in a transferrin (Tf)-bound form, via an endocytic mechanism that involves transferrin receptors (TfRs). Thus far, two TfRs isoforms have been identified, TfR1 and TfR2. These proteins are largely non-redundant, performing different functions, because TfR2 cannot replace TfR1 in cells in which the latter has been silenced (Trinder and Baker, 2003). Consistently, experimental evidence highlighted several differences between TfR1 and TfR2. TfR2 has lower affinity (approximately 30-fold less) for iron loaded transferrin

* Corresponding author.
E-mail address: p.g.mastroberardino@erasmusmc.nl (P.G. Mastroberardino).
[1] co-first authors.

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(i.e. holotransferrin) than TfR1 (Kawabata et al., 2000; West et al., 2000). While TfR1 is largely regulated by intracellular iron levels via the iron responsive element-iron regulatory protein (IRE-IRP) mechanism: high iron concentration exposes an instability element in the mRNA 3′ UTR in the mRNA, therefore promoting degradation. Conversely, TfR2 lacks the iron responsive 3′ UTR element and therefore does not directly respond to intracellular iron concentration (Kawabata et al., 2001). Additionally, unlike TfR1, TfR2 cannot bind the regulatory protein HFE (West et al., 2000), which is mutated in patients with hereditary hemochromatosis and negatively regulates TfR1-mediated iron uptake, at least in cell culture experimental systems (Corsi et al., 1999; Gross et al., 1998; Roy et al., 1999). Finally, recent study indicate that TfR2 acts in concert with the hormone hepcidin, the master iron regulator of the body, to maintain systemic iron homeostasis (Drake et al., 2007; Kawabata et al., 2005).

3. The endo-lysosomal system

In general terms, endocytosis refers to the process that leads to internalization of molecules and fluids from the extracellular environment through the invagination of the plasma membrane and subsequent fission of the latter in vesicles, or endosomes. The endocytic process is responsible for transporting biological molecules – or cargos – to their final subcellular compartment. Cargos can then be either recycled via shuttling to the plasma membrane, or degraded in the lysosomal compartment. Consistently with these general functions, the endo-lysosomal compartment has been originally classified in early endosomes (EE), recycling endosomes (RE), late endosomes (LE), and lysosomes (L), which also display different ultrastructural features (see (Huotari and Helenius, 2011) for review). Quantitative estimates of endosomal activity described in early studies clearly illustrate the proportions - and thus the biological importance - of this process. Cargos internalization is in fact very rapid – it requires only in a few minutes - and recycling involves a very large fraction of the plasma membrane. In contrast, transport for degradation to lysosomes represents a relatively smaller proportion of internalized membranes (Besterman and Low, 1983; Steinman et al., 1983). Current knowledge of the mechanisms responsible for transporting a cargo to degradation is reasonably advanced (Henne et al., 2013) and has been excellently reviewed, also in very recent articles, approaching the problem from the perspective of mitochondria PD (e.g. (Plotegher and Duchen, 2017)). Conversely, our understanding of the recycling branch of the endosomal system highly rudimentary (Hsu et al., 2012).

Overall, the endo-lysosomal compartment is a highly active cellular component governing membrane trafficking as well as proteostasis, therefore participating to the regulation of a multitude of biological pathways. Consistently, the endo-lysosomal machinery is essential for life and its dysfunction is associated with pathology or even lethality.

4. Molecular mechanisms of endocytosis

The endosomal machinery must recognize a cargo and its load, for instance transferrin and its receptor, extract these proteins from the plasma membrane via formation of a vesicle, direct the latter to its subcellular destination, and possibly re-shuttle these components to the plasma membrane for recycling. Endosomal protein sorting and trafficking is regulated by the heteropentameric complex retromer, which is composed by two distinct modules, one of which ensures recognition of the cargo complex, while the other is responsible for tubulation i.e. the formation of tubules that operate cargo transport to its destination. The recognition complex is a heterotrimer composed by the proteins VPS26, VPS29, and VPS35. The latter constitutes the backbone used by the other two proteins to bind and form the complex (Burd and Cullen, 2014). The tubulation complex is a heterodimer composed of a subset of Sorting Nexins (Snxs) - Snx1, Snx2, Snx5, and Snx6 - which can be present in variable combinations and are crucial for correct trafficking organization. In addition, other 29 different SNXs have been identified in mammals (Cullen, 2008; Cullen and Korswagen, 2011). The precise role of these proteins has not been fully characterized yet; nonetheless, such abundance points to specialized functions that may regulate endocytosis in a cargo and/or tissue specific fashion. Mechanistically, Snx bind via their phox-homology (PX) domain to the phospholipids phosphatidylinositol-3-phosphate (PtdIns3P) and phosphatidylinositol-3,5-bisphosphate (PtdIns3,5P2), which are enriched in early and late endosomes (Cullen and Korswagen, 2011).

5. Mitochondria

Generation of ATP via oxidative phosphorylation (OXPHOS) - i.e. electron transport between respiratory complexes to generate a proton gradient across the inner mitochondrial membrane that will be used as thermodynamic driving force for the ATP synthase - is the principal function of mitochondria (Nicholls and Ferguson, 2013). Importantly, other functional aspects of mitochondria, for instance calcium buffering and production of reactive oxygen species to operate redox signaling, also depend on electron transport and proton gradient maintenance (Murphy, 2009).

Iron is particularly important for mitochondrial biology. Electron transport, in fact, is possible because of iron-containing prosthetic groups such as heme and iron-sulfur clusters (ISCs), which serve different purposes: from redox reactions to oxygen transport, to structural functions (Braymer and Lill, 2017). Of note, the biosynthesis of heme occurs exclusively in mitochondria and ISCs are prevalently assembled in these organelles (Braymer and Lill, 2017). Because of these particular features, mitochondria are the major recipient of iron in the cell.

How iron enters mitochondria, however, is highly debated. Given free iron propensity to engage in potentially detrimental redox reactions with products of mitochondrial metabolism (e.g. O_2 or H_2O_2), it is highly unlikely that iron transfer occurs independently from chaperone proteins or mechanisms. Two proteins functioning as uniporter involved in mitochondrial iron uptake – mitoferrin 1 and mitoferrin-2 - have been identified thus far (Paradkar et al., 2009; Shaw et al., 2006) and recent evidence indicates that mitoferrin 1 functions as uniporter transporting unchelated ferrous iron (Christenson et al., 2018). There is large consensus, however, that mitochondrial iron import cannot solely rely on these protein; yet, our understanding of alternative mitochondrial iron import mechanisms is highly rudimentary.

6. Endocytosis in the brain

Neurons are characterized by extremely arborized processes that can reach considerable length and therefore display unique cytological complexity. Additionally, neurons are post-mitotic cells and must survive for the entire lifespan of an organism, which in humans reaches almost one hundred years. Neurons therefore require particularly robust intracellular trafficking machinery and quality control mechanisms, which are reflected in the distinctive cyto-architecture of their endo-lysosomal system. The latter is in fact highly compartmentalized, with different classes of endo- lysosomes located in different cytological domains. For instance, while late endosomes are rather homogeneously distributed, lysosomes with high degradative capacity are typically located in the neuron soma or in proximal neurites and dendrites. A the molecular level, expression of degradative lysosomal markers such as hydrolases manifest progressively as the endosomes are transported toward the soma (Winckler et al., 2018).

During development, migration of neurons is essential for proper function of the adult brain and, when defective, is associated to conditions such as epilepsy and mental retardation. The process is also mediated by membrane receptors and by their appropriate endocytic intracellular trafficking (Yap and Winckler, 2015). Consistently, deletion of essential components of the endocytic machinery – for instance retromer - results in embryonic lethality (Schwarz et al., 2002).

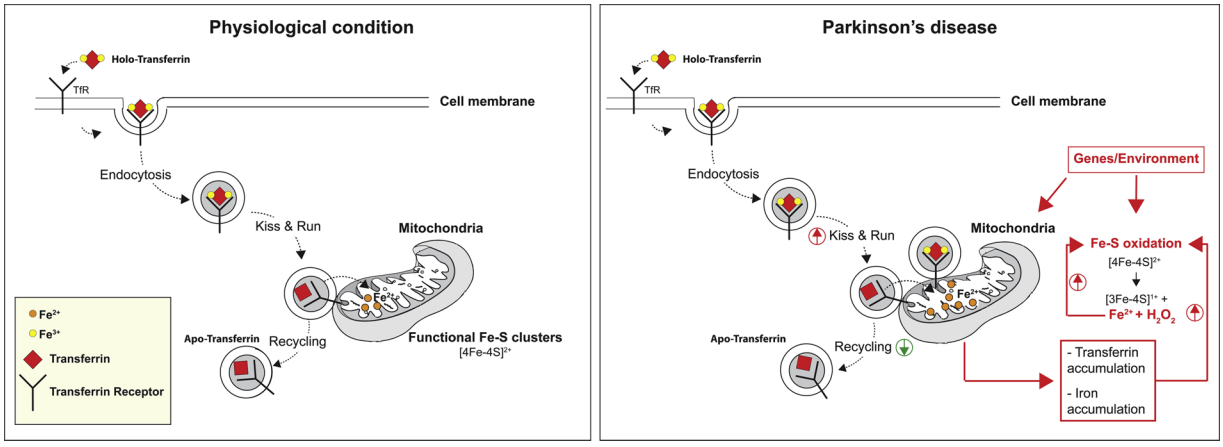

Fig. 1. Schematic summarizing the interactions between endosomes and mitochondria in healthy conditions and in PD. Endosomes may physiologically contribute to iron import in mitochondria via a transferrin-mediated kiss-and-run mechanism (left panel). In PD, oxidation of iron-sulfur clusters may represent a stimulus to increase iron import (right panel). Oxidative stress may prevent proper endosomes' recycling, which can further increases intracellular iron levels. Collectively, these phenomena trigger a vicious circle escalating oxidative stress and promoting pathogenesis.

Adequate endocytosis is also critical for the adult brain, as evidenced by the effects of mutation in genes on the endocytic pathway. Studies based on exome sequencing, in fact, identified genetic association between the VPS35(p.D620 N) mutation and PD (Vilarino-Guell et al., 2011; Zimprich et al., 2011). Other follow up studies identified additional non-synonymous mutations (reviewed in (McMillan et al., 2017). Thus far, available evidence indicates that none of the mutations that have been analyzed at mechanistic level drastically alters the retromer complex and result only in mild trafficking defects (Follett et al., 2016; McMillan et al., 2016, 2017). Moreover, post-mortem brains of idiopathic PD patients fail to exhibit alterations in VPS35 subcellular distribution, despite viral-mediated overexpression of VPS35(p.D620 N) induces dopaminergic degeneration in laboratory rodent models (Tsika et al., 2014). Further complexity arises from a study also identified healthy carriers, therefore suggesting incomplete penetrance of this mutation (Sharma et al., 2012).

Although the lack of consensus on the mechanisms explaining the role of VPS35 in PD pathogenesis, multiple lines of evidence substantiate the relevance of retromer in PD. For instance, dopaminergic signaling through the dopamine D1 receptor (D1R) – and thus through the direct striatonigral pathway - is contributed by rapid D1R endocytosis (Kotowski et al., 2011). Moreover, several independent reports indicate biological interaction between the kinase LRRK2 – the principal genetic risk factor for PD - and VPS35. For instance, in animal models, wild-type VPS35 can rescue *Lrrk2* associated defects and overexpression of the mutated VPS35 form alters the normal *Lrrk2* associated phosphoproteome pattern (Fujimoto et al., 2018; Inoshita et al., 2017; Linhart et al., 2014; MacLeod et al., 2013; Mir et al., 2018). The latter evidence further corroborates the nexus between PD, endocytosis, and LRRK2, because its phosphorylation targets are abundantly represented by a subset of Rab GTPases (Steger et al., 2016), which are fundamental players in the endocytic process (Pfeffer, 2017). A further potential nexus with PD emerges from data showing that overexpression of wild-type VPS 35 is protective against the PD-related mitochondrial toxins MPTP and rotenone (Bi et al., 2013; Linhart et al., 2014). Despite the latter evidence highlights a connection between endocytosis and mitochondria, endocytic trafficking toward these organelles is still poorly understood.

It has been shown that VPS35 can influence mitochondrial dynamics – possibly in a detrimental way in the case of PD associated mutations- even though evidence is not conclusive given that two different studies reported opposite effects, i.e. favoring mitochondrial fission (Wang et al., 2016) or fusion (Tang et al., 2015). An unbiased screen, however, found that VPS35 localizes to mitochondria, where it can direct endocytic trafficking (Braschi et al., 2010). These results are consistent with previous findings from our laboratory demonstrating that Tf can be targeted to mitochondria via TfR2, through a mechanism that can possibly involve an active mitochondrial targeting sequence at the N-terminal of the latter protein (Mastroberardino et al., 2009). Direct endocytic trafficking of Tf/TfR1 to mitochondria has been initially proposed by pioneering studies in erythroid cells (Isobe et al., 1981; Richardson et al., 2010; Sheftel et al., 2007), via a transient "kiss and run" mechanism. A further and more recent independent study used super-resolution microscopy to confirm this evidence in non-erythroid cells (Das et al., 2016). Importantly, these studies also indicate that the Tf/TfRs endocytic pathway can deliver iron to mitochondria. It should be emphasized, however, that the mechanisms mediating endosomal docking onto mitochondria are unknown.

Endosomal trafficking to mitochondria may be of particular relevance for PD because evidence gathered in rodents, non-human primates, and patients show that pathogenesis and iron accumulation are accompanied by increased Tf levels in dopaminergic neurons (Mastroberardino et al., 2009). Iron uptake via the endocytic pathway may therefore be a significant route of iron accumulation even though it is not known yet whether PD patients carrying mutations in the endocytic protein VPS35 also display iron accumulation, and, if so, to which extent (Funke et al., 2013).

Mechanistically, iron uptake may be driven by increasing oxidative stress in DA neurons, which occurs during PD progression (Mastroberardino et al., 2009, 2008). Oxidant species may oxidize ISCs, for instance from $[4Fe\text{-}4S]^{2+}$ to the inactive $[3Fe\text{-}4S]^{1+}$, with consequent decrease in the number of functional mitochondrial proteins relying on these prosthetic groups (Gardner et al., 1995; Vasquez-Vivar et al., 2000). Here, it should be noted that oxidation of $[4Fe\text{-}4S]^{2+}$ to inactive $[3Fe\text{-}4S]^{1+}$ produces ferrous iron and hydrogen peroxide therefore lay foundation for potentially detrimental redox cycling (Vasquez-Vivar et al., 2000). It has also been proposed that ISCs in proteins may act as sensors of mitochondrial iron status and chemical or genetic depression of ISC or heme synthesis alters Tf and iron biology, even though with counterintuitive consequences. In fact, one would think that such depression should in principle reduce mitochondrion iron need; it sorts, instead opposite effects, causing increased uptake of Tf-bound iron, which culminates in mitochondrial iron overload (Huang et al., 2009). The mechanisms driving this process are obscure, but iron uptake despite apparent lack of necessity (i.e. without heme or ISC biosynthesis) is generally interpreted as an attempt of the cell to rescue the synthesis of these important molecules. Accordingly, ISC oxidation is may be reversed in ferrous iron excess (Vasquez-Vivar

et al., 2000). On these premises, Tf increase observed in the rotenone model of PD and in patients' specimens can be interpreted as a consequence of ISC oxidation – which is supported by biochemical evidences *in vitro* and *ex vivo* (Flint et al., 1993; Panov et al., 2005) – leading to increased TfR cycling rate to promote ISC synthesis. When trafficking is mediated by TfR2, which does not contain an IRE and is therefore not directly regulated by iron concentration, increased Tf uptake under oxidative stress conditions may further aggravate iron overload (Fig.1).

7. Conclusions

A role for alterations in endocytosis in PD pathogenesis is rapidly gaining momentum. Endocytic trafficking connects critical aspects of PD pathobiology, such as mitochondrial defects, iron mishandling, and oxidative stress, and it might therefore constitute a tractable target for future therapeutic interventions.

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