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Iron dyshomeostasis in Parkinson's disease

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Summary Owing to its ability to undergo one-electron reactions, iron transforms the mild oxidant hydrogen peroxide into hydroxyl radical, one of the most reactive species in nature. Deleterious effects of iron accumulation are dramatically evidenced in several neurodegenerative diseases. The work of Youdim and collaborators has been fundamental in describing the accumulation of iron confined to the substantia nigra (SN) in Parkinson's disease (PD) and to clarify iron toxicity pathways and oxidative damage in dopaminergic neurons. Nevertheless, how the mechanisms involved in normal neuronal iron homeostasis are surpassed, remain largely undetermined. How nigral neurons survive or succumb to iron-induced oxidative stress are relevant questions both to know about the etiology of the disease and to design neuroprotective strategies. In this work, we review the components of neural iron homeostasis and we summarize evidence from recent studies aimed to unravel the molecular basis of iron accumulation and dyshomeostasis in PD.

In vertebrates, numerous physiological processes including oxygen transport, respiration, DNA synthesis, formation of some neurotransmitters and hormones, xenobiotic metabolism, and certain aspects of host defense use iron-containing proteins (Gutteridge and Halliwell, 2000). However, because of its ability to undergo one-electron reactions, Fe^{2+} transforms the mild oxidant hydrogen peroxide into hydroxyl radical ($HO^{\bullet}$), one of the most reactive species in nature (Symons and Gutteridge, 1998). The Fenton reaction, as this reaction is known, follows mass action law, so $HO^{\bullet}$ production is proportional to reactive Fe^{2+} concentration. There are no known specific mechanisms to detoxify $HO^{\bullet}$, so this species quickly reacts and modifies lipids, proteins, lipids and DNA (Gutteridge and Halliwell, 2000; Haupmann and Cadenas, 1997). To maintain iron inside a concentration window that allows for physiological functions, and impedes the formation of highly reactive oxygen species (ROS), cells display transcriptional and post-transcriptional mechanism of regulation.

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Neuronal iron homeostasis

Components of cell iron homeostasis

The components of cell iron homeostasis are shown in Fig. 1. The scheme includes the inflow and efflux iron transporters, DMT1 and Ireg1, respectively; the iron storage protein ferritin; the ferrireductase Dcytb, responsible for the reduction of Fe^{3+} prior to transport by DMT1 (McKie et al., 2001); and the ferroxidase ceruloplasmin, responsible for the oxidation of Fe^{2+} after transport by Ireg1 and prior to the binding by apoTf (Hellman and Gitlin, 2002). The cell uptake system includes the endocytosis of the iron-binding protein transferrin (Tf) mediated by transferrin receptors (TfR) located in the cell surface.

The labile iron pool (LIP)

The LIP is defined as a pool of weakly bound iron. When determined by calcein fluorescence quenching, the affinity constant of LIP complexes is operationally defined as $<10^6$ (Epsztejn et al., 1997). Because of the reductive environment of the cell, iron in the LIP is predominantly in the Fe^{+2} state, although transient Fe^{+3} is expected because of cellular oxidations. The nature of the LIP binding counterpart is unknown, but has been ascribed to diverse low-molecular weight substances as phosphate, nucleotides, hydroxyl, amino and sulphydryl groups (Kakhlon and Cabantchik, 2002; Petrat et al., 2002). Normally, the LIP represents 3–5% of the total cellular iron, but this proportion changes with the iron status of the cell. Neuroblastoma

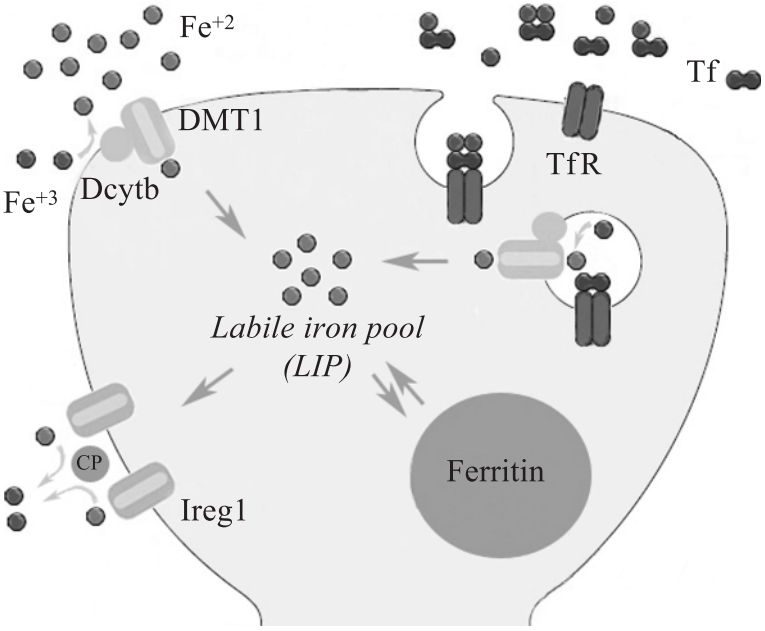

Fig. 1. Neuronal Iron Homeostasis. Neurons may acquire iron from two sources, Tf-bound iron and non-Tf-bound iron (NTBI). The total iron content and Tf concentration in CSF (about 0.7 and 0.24 μM, respectively) ensures that both sources of iron are readily available to brain cells. Tf- bound Fe is taken up via a TfR-mediated process. This process involves the binding of diferric Tf to TfR, the internalization of the complex into an acidic endosomal compartment, which promotes the dissociation of iron from Tf. Dissociation is followed by the reduction of released Fe^{3+} to Fe^{2+} and the transport into the cytosol through DMT1. NTBI uptake involves the reduction of Fe^{3+} to Fe^{2+} by Dcytb and the transport by DMT1. The high content of ascorbate in CSF (about 150 μM) ensures that CSF iron will be mainly in the Fe^{2+}, readily transportable state. Once in the cytosol, Fe^{2+} incorporates in to the labile iron pool, where it establishes multiple equilibriums with other iron-containing compartment in the cell, the quantitatively most important of them being ferritin. Recent reports indicate that iron exit loaded neurons (Aguirre et al., 2005). Iron transporter Ireg1 is the most probable candidate to mediate this transport, since its increased expression due to iron loading closely correlates with the rate of iron efflux (Aguirre et al., 2005). After Ireg1 transport, and prior to the binding with Tf, Fe^{2+} is oxidized to Fe^{3+}. The oxidation is mediated by ceruloplasmin present in CSF. Abbreviations: DMT1, divalent metal transporter 1; Ireg1, iron-regulated transporter 1; Dcytb, duodenal cytochrome b ferrireductase; CP, ceruloplasmin; Tf, transferrin; TfR, transferrin receptor; FN, ferritin

cells cultured with high iron media contain 3-4-fold more LIP that cells cultured in low iron media (Núñez et al., 2004). Moreover, cells with higher LIP invariably present higher levels of reactive oxygen species. Thus, the LIP is a marker not only of total cell iron content but also determines the redox state of the cell (Kruszewski, 2003; Núñez et al., 2004).

Over 90% of cell iron is safely stored in ferritin, a multimeric protein that accept up to 4,500 iron atoms in its central core. Since ferritin iron is not redox-active, it is considered ''safe'' iron. Nevertheless, ferritin iron is not completely safe. Every time ferritin is degraded (microglial ferritin turnover: 3–3.5 h (Mehlhase et al., 2005), its iron will contribute transiently to the LIP. Immunohystochemical studies of ferritin distribution in brain cells show a ubiquitous label for oligodendrocytes and microglia and a weak mark in neurons from cortex, medial habenula, paraventricular nucleus, amygdale and supraoptic nucleus (Connor et al., 1994; Hansen et al., 1999). Thus, we might conjecture that in the majority of neurons, iron uptake and export would be more important than ferritin turnover in determining LIP.

Tf-mediated iron uptake

Tf is present in extracellular fluids either in the apo, monoferric or diferric forms. Under physiological conditions, iron enters the cell by Tf endocytosis. In the acidic pH of the endosome, iron is released from Tf, reduced to Fe^{2+} by a ferric reductase and transported to the cytosol (Núñez et al., 1990). Once in the cytosol, Fe^{2+} becomes part of LIP (Breuer et al., 1997). Plasma Tf saturation in normal individuals is about 35%, which makes monoferric Tf the predominant plasmatic form (Williams and Moreton, 1980). Tf saturation is different in brain cells. Cerebral spinal fluid (CSF) Tf and iron concentrations are about 0.24 and 0.70 μM, respectively (Symons and Gutteridge, 1998). Thus, cerebral spinal fluid Tf is saturated and considerable amounts of non-Tf-bound iron are present. Providing the presence of DMT1 or TfR in the plasma membrane, brain

cells will carry out both transferrin-bound and non transferrin-bound iron uptake.

The iron transporter DMT1

DMT1 transports iron into cells by an electrogenic mechanism that involves the co-transport of Fe^{2+} and one proton. In the brain, DMT1 messenger and protein are present mainly in neuronal cells (Gunshin et al., 1997). The highly reductive property of CSF, contributed by ascorbate concentrations of approximately 160 μM (Reiber et al., 1993), determines that Fe^{2+} is the predominant Fe species. Fe^{2+} is readily available for direct incorporation into the cell by the Fe^{2+} transporter DMT1 (Gunshin et al., 1997; Arredondo et al., 2003) in a process known as non-transferrin bound iron uptake. Importantly, non-transferrin bound iron uptake does not have the tight regulation of transferrin-bound iron uptake, exerted by the IRE/IRP system over TfR translation. Thus, neurons with high expression of DMT1 should be particularly susceptible to iron accumulation. In particular, neurons from ventral mesencephalon have one of the highest DMT1/TfR expression ratios (Gunshin et al., 1997; Hill et al., 1985), so these neurons should be prone to iron accumulation.

In searching for DMT1 expression in the rat mesencephalon, we found that co-localization of DMT1 with the dopaminergic marker tyrosine hydroxylase (TH) is higher in the SNpc than in the ventral tegmental area (VTA) (Fig. 2). We hypothesize that he presence of high levels of DMT1 in SNpc might explain its higher susceptibility to neurodegenerative processes.

Four isoforms of DMT1 are generated by alternative splicing of the 5′-end exons (exons 1A or 1B) and of the 3′-end exons (exons 16 (or +IRE) or 16a (or −IRE)) (Hubert and Hentze, 2002). Concordant with the presence of an IRE element in the 3′flanking region, DMT1 expression is down regulated by high cell iron. Nevertheless, there is no agreement about the specific mechanisms causing this regulation. Expression of the 1A/+IRE isoform makes cells particularly sensitive to cell iron levels; expression of the 1A/−IRE isoform yield cells that also responds to iron changes, whereas cells expressing the 1B/+IRE or the 1B/−IRE isoforms do not respond. Thus, it is possible that the regulation of DMT1 expression involves two regulatory regions, one contained in exon 1A and another in exon 16. There is no report on the nature of the DMT1 isoforms present in different brain areas. This information is of primary interest to understand regulation of DMT1 expression.

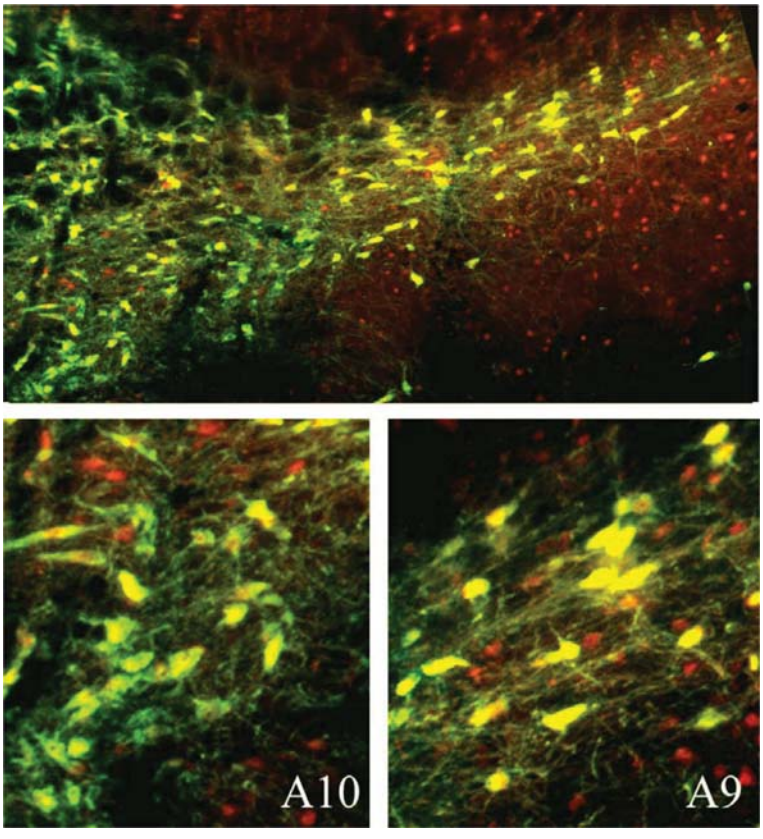

Fig. 2. DMT1 localization in the ventral mesencephalon. Immunohistochemistry against tyrosine hydroxylase (TH, green) and DMT1 IRE+ (red) in a coronal slice of rat mesencephalon. A9, substantia nigra pars compacta. A10, ventral tegmental area. In TH-positive cells, DMT1 immunoreactivity was more intense in A9 than in the A10 area

Iron export

Ireg1 (also called ferroportin 1 and MTP1) is the only member of the SLC40 family of transporters and the first reported protein that mediates the exit of iron from cells (McKie et al., 2000). The protein is expressed mainly in enterocytes and macrophages. In enterocytes, Ireg1 is responsible for iron efflux during the process of intestinal iron absorption, while in Kupffer cells Ireg1 mediates iron export for reutilization by the bone marrow (Devalia et al., 2002). The regulation of Ireg1 expression is unknown. In enterocytes, iron deficiency induces Ireg1 expression (McKie et al., 2000) whereas in macrophages iron deficiency decreases it (Yang et al., 2005). In the brain, we can find Ireg1 messenger and protein in endothelial cells of the blood–brain barrier, in neurons, oligodendrocytes, astrocytes, the choroid plexus and ependymal cells (Wu et al., 2004). In a recent study we reported that SHSY5Y neuroblastoma cells and hippocampal neurons that survive an iron accumulation protocol, evoke an adaptive response consisting of decreased synthesis of DMT1 and increased synthesis of Ireg1 (Aguirre et al., 2005). Thus, the concerted regulation of iron transporters is clearly cell-specific and adjusts to the particular functions of the cells.

The iron reductase Dcytb

Before iron uptake via the membrane transporter DMT1, ferric iron reduction to ferrous iron is attained by the enzyme duodenal cytochrome b (Dcytb) (McKie et al., 2001). Dcytb is also expressed in spleen and probably liver (Latunde-Dada et al., 2004), but no report on the expression of Dcytb in brain cells is available. It is possible that the highly reductive environment of CSF makes redundant Dcytb expression for non-transferrin iron uptake. Nevertheless, Tf-bound iron uptake still should require a Fe^{3+} to Fe^{2+} reduction step before transport into the cytosol (Núñez et al., 1990). Thus, the presence of Dcytb, or a related reductase, in endosomal compartments of brain cells is possible.

The iron oxidases

Two ferroxidases has been described to account for Fe^{2+} oxidation prior to Tf binding, the membrane-bound oxidase hephaestin and serum ceruloplasmin. Hephaestin is a membrane-bound ferrireductase homologous to ceruloplasmin, highly expressed throughout the small intestine, and at low levels in several other tissues (Anderson et al., 2002). No role for hephaestin in human neuronal iron efflux has been demonstrated, thus in the brain, the main ferroxidase involved in iron exit seems to be ceruloplasmin. This member of the multicopper oxidase family exists in the brain predominantly as a glycosylphosphatidylinositol-linked astrocyte protein (Patel and David, 1997). Aceruloplasminemia, a condition characterized by the complete lack of ceruloplasmin ferroxidase activity caused by mutations in the ceruloplasmin gene, results in excessive iron accumulation in the pancreas, retina, and brain (Miyajima et al., 2003). Magnetic resonance imaging of an aceruloplasminemia patient revealed abundant iron deposition in neurons of the putamen, dentate nuclei, substantia nigra, red nuclei, inferior and superior colliculi, and thalamic nuclei (Grisoli et al., 2005). These data can be interpreted as the need of several neuronal areas to discharge iron in order to avoid excessive accumulation.

Iron regulatory proteins: translational regulation

In vertebrates, cellular iron levels are post-transcriptionally controlled by the activity of iron regulatory proteins (IRP1 and IRP2), cytosolic 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: the transferrin receptor, involved in plasma-to-cell iron transport, and the iron-storage protein ferritin (reviewed in Eisenstein and Ross, 2003). The activities of both IRP1 and IRP2 respond to changes in cellular Fe through different mechanisms. In iron-replete conditions, IRP1 has a 4S-4Fe cubane structure that renders the protein active as a cytosolic aconitase but inactive for IRE-binding. Low levels of intracellular Fe induce disassembling of the 4S-4Fe cluster, which causes IRP1 to bind and stabilize TfR mRNA. Furthermore, IRP1 binds to ferritin mRNA, thus diminishing its translation. Besides iron, effectors such as nitric oxide (Bouton et al., 1997; Kim and Ponka, 2002), hydrogen peroxide (Martins et al., 1995), hypoxia (Hanson et al., 1999), and phosphorylation (Schalinske and Eisenstein, 1996) also regulate IRP1. In contrast to IRP1, IRP2 activity is down-regulated through iron-induced oxidative damage followed by ubiquitination and proteasome degradation (Guo et al., 1995).

Iron accumulation in neurodegeneration

Transition metals such as iron and copper are frequently associated with neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease (PD) and Amyotrophic lateral sclerosis (ALS) (Gerlach et al., 1994; Sayre et al., 2000; Perry et al., 2003).

Iron accumulation in Parkinson's disease

Iron accumulates with age in redox-sensitive tissues such as *substantia nigra pars compacta* (SNpc) (Sofic et al., 1988; Dexter et al., 1989; Riederer et al., 1989). Postmortem studies describe higher levels of iron in normal SNpc than in other brain regions and a further iron accumulation in SNpc of patients with PD (Riederer et al., 1989; Youdim and Riederer, 1993; Shoham and Youdim, 2002; Gotz et al., 2004; Hirsch et al., 1991). This has been confirmed by MRI imaging and ultrasound studies (Gorell et al., 1995; Berg et al., 1999). This increase in iron levels is also present in animal models of PD using the neurotoxins N-methyl-1,2,3,6 tetrahydropyridine (MPTP) or 6-Hydroxydopamine (6-OHDA). In sum, these findings led to the hypothesis that excess iron is the cause of dopaminergic neuronal death in PD (Double et al., 2000). Nevertheless, the position of iron accumulation in the cascade of events that trigger PD has been object of intense debate. Is this accumulation a primary event? PD patients with mild loss of dopaminergic neurons and patients with incidental Lewy body disease (ILBD), postulated as pre-symptomatic PD, present with no significant changes in iron levels in SN (Riederer et al., 1989; Dexter et al., 1994). In addition, in MPTP-treated monkeys, dopaminergic cell death precedes iron elevation (He et al., 2003). However, a causative association cannot be discarded since the status of the LIP in early stages of the disease is not known. However, it seems clear that iron contributes to the disease progression. Recent works report that iron chelation protects against MPTP and 6-OHDA neurotoxicity (Kaur et al., 2003; Youdim et al., 2004), and several pre-clinical trials are already in course to evaluate iron chelators that can cross the haematoencephalic barrier as neuroprotective agents (Youdim et al., 2005).

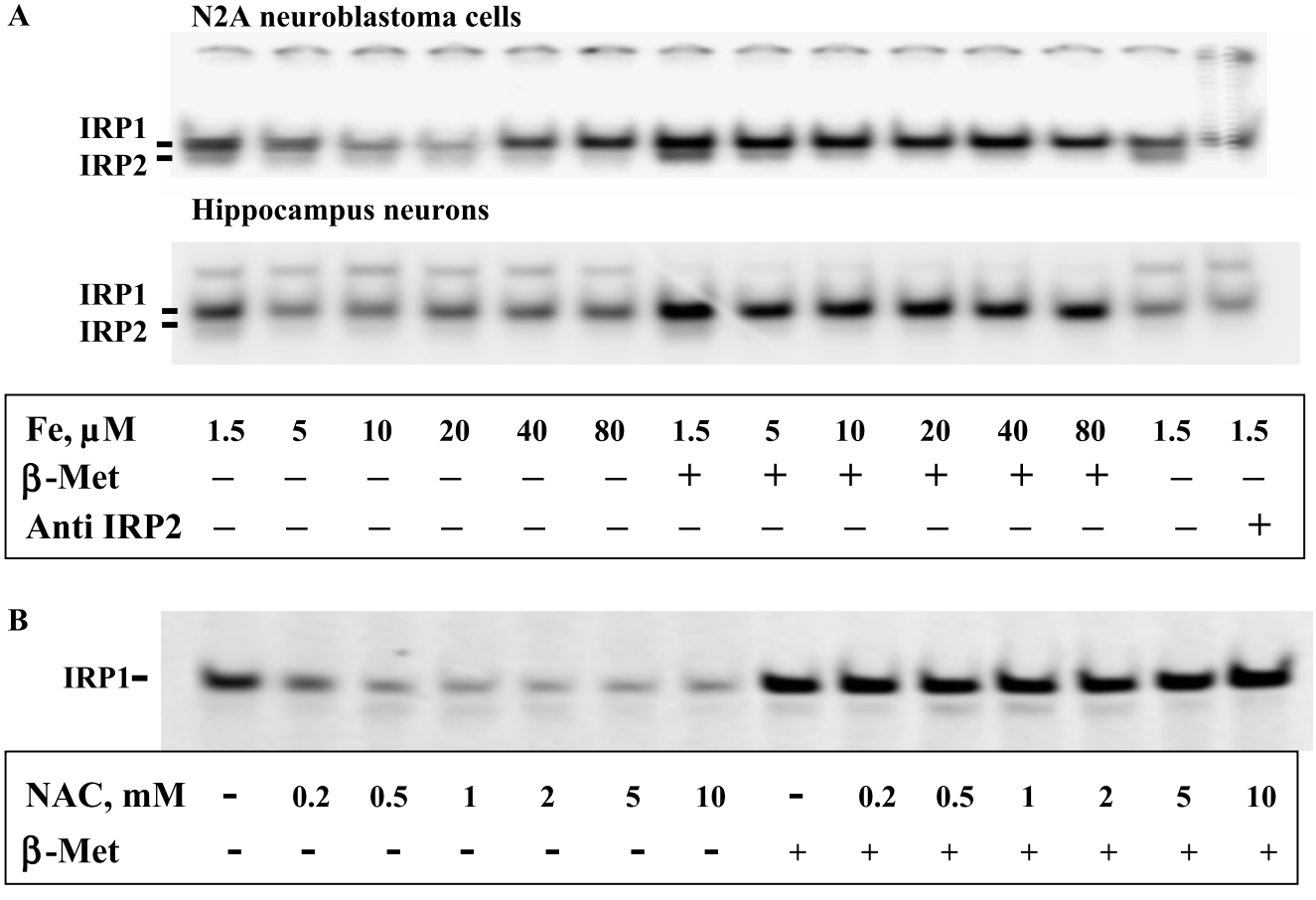

Fig. 3. Effect of iron and antioxidants on IRP activity of neuronal cells. **A** N2A neuroblastoma cells or hippocampal neurons were incubated for 2 days in a media containing 1.5, 5, 10, 20, 40, or 80 µM Fe. Cell extracts were prepared and IRP activity was determined by band-shift assay, using ^{32}P-labeled IRE derived from ferritin cDNA (Núñez-Millacura et al., 2002). The high molecular weight band represents the IRP1-^{32}P-IRE complex, an expression of IRP1 binding activity. Treatment with 2% β-mercaptoethanol (β-Met) maximally activates IRP1, giving a notion of total (active + inactive) IRP1. Following an initial decrease in IRP1 activity as a function of increasing iron, further increases resulted in sustained IRP1 activity. **B** Band-shift assay of IRP activity of N2A cells stimulated for 2 days with 40 µM Fe in the presence of the stated concentrations of the antioxidant N-acetyl-L-cysteine (NAC). It is observed that NAC abolishes the sustained iron-induced IRP1 activity (Fig. adapted from Núñez-Millacura et al. (2001))

We do not know what causes iron accumulation and the factors responsible for overcoming the iron regulatory system. Diferric transferrin binding sites are decreased on melanized neurons of SN (Faucheux et al., 1997). IRP1 activity and the amount of H-Ferritin and L-Ferritin messengers and proteins, present no significant differences between the SNpc of PD patients and control subjects (Faucheux et al., 2002).

An association between ROS/RNS toxicity and PD was found in studies describing: (i) oxidative damage to DNA, lipids and proteins in the SNpc of patients with PD (Alam et al., 1997; Dexter et al., 1989; Floor and Wetzel, 1997); and (ii) NO damage to proteins in Lewis body containing neurons (Good et al., 1998). Thus, considering modulation of IRPs activity by ROS and RNS, it is possible that free radicals in PD impede the expected inactivation of IRP1 at high iron concentrations.

In neuroblastoma cells and cultured neurons, the activity of IRP1 in function of iron concentration shows a biphasic curve: a first component in which iron IRP1 decreases as iron augments, and a second component in which IRP1 increases with further rise in iron concentration (Núñez-Millacura et al., 2002). Interestingly, the upward component is dependent on oxidative stress, since it is reversed by the anti-oxidant N-acetyl cysteine (Fig. 3). These results led us to think that, even when dopaminergic cell might be trying to compensate iron accumulation, globally, the iron-induced oxidative stress maintains IRP1 active, with the consequent failure to up-regulate ferritin (Núñez-Millacura et al., 2002).

A role for inflammation in PD is supported by microglial activation, cell infiltration and augmented cytokines reported in the nigrostriatal system of parkinsonian brains (Hirsch et al., 2003). Conspicuously, studies in monocytic cells, bronchial epithelial cells and endothelial cells showed increased levels of DMT1 and/or decreased level of Ireg1/ferroportin as a consequence of exposition to lipopolysaccharide, TNFα or INFγ (Ludwiczek et al., 2003; Wang et al., 2005; Nanami et al., 2005). Furthermore, NFκB augments the transcription of DMT1 1B (Paradkar and Roth, 2005), and nuclear immunoreactivity of NFκB in PD brains was reported to be over 70-fold that of control subjects (Hunot et al., 1997). To our knowledge, there is no report of the status of DMT1 and Ireg1 expression in SN of PD brains. We hypothesize that dopaminergic cells, which abundantly express TNFα receptors (Boka et al., 1994), will be prone to have increased iron content by the unfavorable expression of iron import and export transporters (Aguirre et al., 2005).

Recent works have revealed that ubiquitin-proteasome system, the main pathway of cellular protein degradation, is specifically impaired in the SN of parkinsonian brains (McNaught and Olanow, 2005). Given that IRP2 is degraded by this system, its impairment could favors IRP2 accretion and increases of iron. Although, band shift assays have failed to find IRP2 activity in human brains, we can search for changes of protein levels.

Iron regulatory proteins and DMT1 in an animal model of Parkinson's disease

In order to test a putative activation of IRP1 and DMT1, we examined IRP activity and protein content of DMT1 in an animal model of PD. We selected the model of partial lesion with striatal injection of 6-OHDA (20 μg/5 μl for

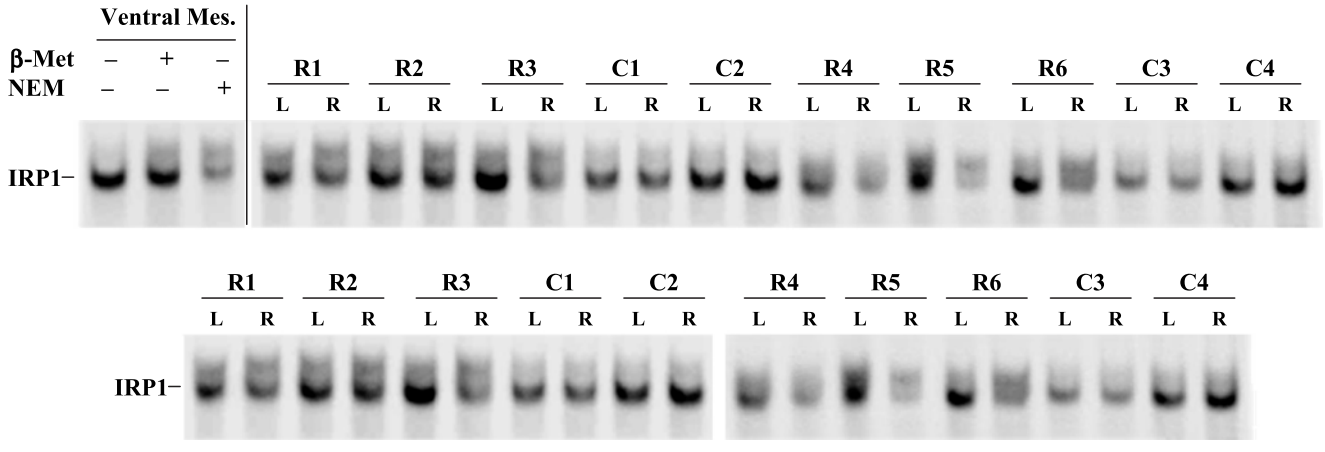

Fig. 4. IRP activity in ventral mesencephalon from 6-OHDA treated rats. IRP activity was determined in cell extracts of ventral mesencephalon It is observed that IRP1 activity is higher in the ventral mesencephalon ipsilateral to the side of 6-OHDA injection (L) in all experimental rats (R1–6). No differences where found between both sides in sham operated rats (C1–4)

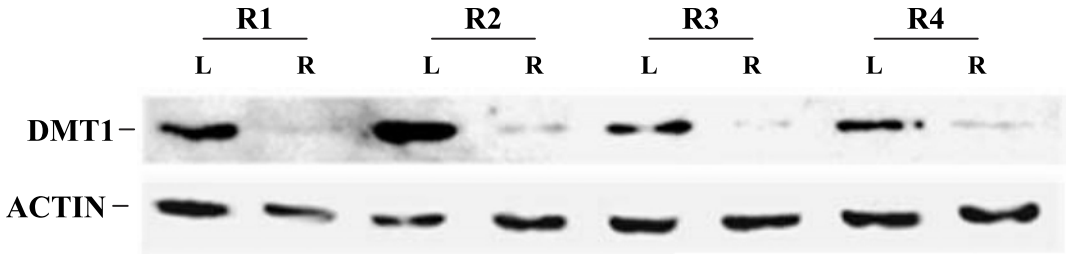

Fig. 5. DMT1 expression in ventral mesencephalon from 6-OHDA treated rats. Ventral mesencephalon from 6-OHDA-treated or control rats was dissected and proteins resolved by SDS-PAGE electrophoresis. DMT1 was recognized by Western immunoblotting using an antibody that recognizes the +IRE isoform of DMT1. To control for gel load, the membranes were acid-stripped after the detection of DMT1 and re-probed with anti-actin (Aguirre et al., 2005). The intensity of the DMT1 band was higher in the ventral mesencephalon ipsilateral to the side of 6-OHDA injection (L) in all experimental rats (R1–4). No differences where found between both sides in sham operated (data not shown)

animal in one injection) because microglial activation and cytokines rise have been reported for this model (Henze et al., 2005; Mogi et al., 1999). In addition, unilateral injections offer the possibility of a control side. At 21 days post-injection, SN of treated rats presented a 40% loss of tyrosine hydroxylase (TH)-positive cells when compared with the control side. Total iron levels were 36% augmented in the SN ipsilateral to the lesion compared with the control side, as measured by mass spectrometry. The ventral mesencephalon (containing the SN) of accurately lesioned rats was dissected and tested in band-shift assays for IRP activity (Fig. 4) and western blot for DMT1 protein levels (Fig. 5). We found an augmentation in both IRP1 activity and in DMT1 protein levels in the SN of all lesioned animals. These finding points to a severe dysregulation of iron homeostasis in 6-OHDA animals. It remains to be determined what is first: unchecked iron accumulation and a positive feedback loop of oxidative stress and IRP1/DMT1 dysregulation or IRP1/DMT1 dysregulation induced by an unknown process resulting in iron accumulation. If increased DMT1 activity is a pathognomonic sign of iron dysregulation, we predict that animals presenting a mutated DMT1 transporter, such as the Belgrade rat and the *mk*-mouse, would present with less susceptibility to neurotoxins causing nigral degeneration.

Concluding remarks

In PD, the iron regulatory mechanisms in neurons of SNpc are surpassed. Recent evidence suggests that IRP1-mediated iron dyshomeostasis and sustained DMT1 presence may underlie iron accumulation in this disease. In this context, an evaluation of DMT1 and Ireg1 expression in parkinsonian brain is capital to understand the cause of iron accumulation and to design neuroprotective and therapeutic strategies to prevent progression of PD.

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