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Review

Copper, iron, and zinc ions homeostasis and their role in neurodegenerative disorders (metal uptake, transport, distribution and regulation)

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Contents

1. Introduction 2666
2. Oxidative stress, mitochondrial dysfunction, aging and neurodegeneration 2666
3. Copper 2667
   3.1. Copper homeostasis 2667
   3.2. The copper metabolism protein MURR1/COMMD1 2670
   3.3. Metallothioneins 2672
   3.4. Toxicity of Cu and its role in apoptosis 2673
   3.5. Copper in neurodegeneration 2674
      3.5.1. Copper and the amyloid precursor protein (APP) 2674
      3.5.2. Copper and the prion protein 2675
4. Iron 2676
   4.1. Iron homeostasis 2677
   4.2. Iron in brain aging and neurodegenerative disorders 2678
5. Zinc 2680
   5.1. Zinc homeostasis 2680
   5.2. Zinc in brain aging and neurodegenerative disorders 2681
6. Conclusions 2682
   Acknowledgement 2682
   References 2682

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ABSTRACT

Metal ions, especially with high chemical activity (e.g. redox-active Cu and Fe) must be carefully managed in biological systems. The "uncontrolled" activity, e.g. catalysis of Fenton-like reactions by ions like Cu(I) or Fe(II), is so damaging for the biological milieu that right from their entry, metal ions need to be strictly controlled until they arrive at their storage site. This chaperoning occurs usually by proteins which are

Abbreviations: ABAD, Aβ-binding alcohol dehydrogenase; AD, Alzheimer's disease; AIF, apoptosis-inducing factor; ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; ATP, adenosine-5′-triphosphate; Aβ, amyloid-β; BBB, blood–brain barrier; CCS, copper chaperone for superoxide dismutase; CDF, cation diffusion facilitator; CNS, central nervous system; COMMD1, copper metabolism (Murr1) domain containing 1; Cox17, COX17 cytochrome *c* oxidase assembly homologue; CSF, cerebrospinal fluid; Ctr1, copper transporter 1; Ctr2, copper transporter 2; CuBD, copper-binding domain; Cyt-*c*, cytochrome *c*; DEPC, diethyl pyrocarbonate; DMT1, divalent metal transporter; ETC, electron transport chain; FALS, familial amyotrophic sclerosis; FPN, ferroportin; Fre1, ferric reductase 1; Fre2, ferric reductase 2; FTL, ferritin light polypeptide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPI, glycosyl-phosphatidylinositol; GSH, glutathione; HFE, hemochromatosis gene; IL-1, interleukin 1; IMS, intermembrane space; IRE, iron responsive elements; IRP, iron regulatory proteins; KGDHG, α-ketoglutarate dehydrogenase; LEC, Long–Evans cinnamon; MCO, metal-catalyzed oxidation; mPTP, mitochondrial permeability transition pore; MTs, metallothioneins; NFTs, neurofibrillary tangles; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NMDA, (N-methyl-D-aspartic acid); nNOS, nitric oxide synthase; $ONOO^-$, peroxynitrite; OXPHOS, oxidative phosphorylation; PD, Parkinson's disease; PrP^C, cellular prion protein; PrPD, prion diseases; PrP^{Sc}, scrapie prion protein; ROS, reactive oxygen species; SLC, SoLute carrier; SOD, super oxide dismutase; SP, senile plaques; TGN, trans-Golgi network; VSCCs, voltage sensitive calcium channels; XIAP, X-linked inhibitor of apoptosis; ZnBD, zinc-binding domain; ZnTs, Zn(II) transporter proteins; $[Zn(II)]_i$, intracellular free Zn(II); $\Delta\Psi_m$, mitochondrial membrane potential.

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involved in transport, delivery and distribution processes. In this review some aspects of the metal homeostasis for major metal ions (Cu, Fe, and Zn) are presented. The impact of these metals on some disorders are also discussed.

1. Introduction

More than one-third of all proteins need metal ions for functioning. The metal ion-binding sites are usually very complex and very specific for particular monomeric or polynuclear metal centres [1,2]. It could be rather risky to let the protein select the correct metal ion(s) from the mixture of metals present in the cell using even the most specific set of amino acid donor systems. The binding affinity derived from the set of donor atoms and coordination geometry is not sufficient for the perfect selection of a metal ion. On the other hand, the concentration of the needed metal may be too low or too high and metal export or its sequestering will be required. Even essential metal ions like copper or iron could be highly toxic when badly managed. Cells very efficiently exploit speciation in the metal–protein systems and reach a very strict control over the metal acquisition, distribution and regulation processes.

Copper, zinc and iron are essential trace elements responsible for the function of many cellular enzymes and proteins; however, the same elements become toxic whenever excessive intracellular accumulation occurs. The redox activity seems to play a driving role in addressing the main effects of these trace metals. In fact, copper and iron may contribute to the production of free radicals and, therefore, are likely to play a relevant role in regulation and induction of apoptosis. On the contrary, zinc, either directly or through the induction of metallothionein, is agreed to have protective roles against oxidative damage.

All toxic metals share the common initial feature of generating oxidative stress: iron and copper can directly catalyze the production of hydroxyl radicals from hydrogen peroxide (Fenton reaction) and other reactive oxygen species (ROS) [3], whereas the redox-inactive metals, can diminish the ROS-scavenging capacity in cells, but may also stimulate the generation of ROS indirectly by displacing Fe and Cu from MT and other metal-containing cellular proteins, which then can accelerate the production of ROS via the Fenton reaction. [4] In this context, oxidative stress defined as a disturbance in the prooxidant–antioxidant balance can contribute to the development of potential cell damage and consecutive apoptosis [5].

Oxidative stress is considered one of the many triggers of aging in the brain. Oxidative damage of macromolecules increases with age leading to a progressive decline in cell and tissue function. Mitochondria are one of the major systems that are targeted by aging-related oxidative stress. The aim of this review is to offer a conceptual framework that might help to further unravel the role of copper, iron and zinc in age-related neurodegeneration. The focus of the review is on the relationship between metal dyshomeostasis, mitochondrial dysfunction, oxidative stress, and mitochondrial genomic damage, to determine how the combination of these factors might promote aging of the brain.

2. Oxidative stress, mitochondrial dysfunction, aging and neurodegeneration

Neurodegenerative diseases as diverse as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), the prion diseases (PrPD), and others, all belong to a heterogeneous group of disorders where anatomically or physiologically related neuronal systems are gradually, progressively, and selectively degraded. All these disorders have been known for many years, but, in spite of that, no cure has been successfully suggested so far, and the sufferers gradually and irreversibly lose their independence and require dedicated on-going care, at enormous economic and social cost. Despite the intrinsic heterogeneity, several important common themes in these diseases have been suggested and demonstrated [6]:

- the presence of intra- or extracellular proteinaceous deposits (the amyloid-β (Aβ) plaques and the neurofibrillary tangles (NFTs) of AD, the α-synuclein-containing Lewy bodies of PD, the SOD1-containing Bunina bodies of ALS and the PrP^{sc}-made deposits of PrPD);
- all diseases may be of familial or sporadic origin: no more than 10–12% of the diseases have a strict genetic etiology while majority of cases are sporadic. Only PrPD may also be of infectious origin;
- increased oxidative damage to lipids, proteins and nucleic acids;
- a loss of biometal homeostasis.

The deposition of aggregated proteins causes neuronal damage and contributes substantially to the diverse pathologies [7]. These deposits, however, cannot be regarded as 'up-stream' causative factors, since soluble intermediate oligomers are substantially more toxic than formed fibrils [8]. As a consequence, targeting protein deposits may ameliorate some of the neurological signs and decrease the burden of the diseases, but without any impact on the biological mechanisms that caused them to develop. It is thus emerging that the fundamental research aim is to identify the biological mechanisms that trigger neurodegeneration and, consequently, to develop more effective therapeutic strategies.

From this point of view, the involvement of mitochondria may provide an important common theme that may overcome the intrinsic heterogeneity of all neurological disorders [9]. Aging is in fact by far the greatest risk factor for neurodegenerative diseases, and mitochondria have been thought to contribute to aging through the accumulation of mitochondrial DNA (mtDNA) mutations and net production of reactive oxygen species (ROS). Mitochondria are key regulators of cell survival and death [10], have a central role in aging, and have recently been found to interact with many of the specific proteins implicated in genetic forms of neurodegenerative diseases.

ROS are produced in many cellular compartments throughout the activity of several enzymatic pathways, but the majority of cellular ROS are produced by mitochondria as a toxic by-product of oxidative phosphorylation (OXPHOS). Functional mitochondria exert a critical role in such events, as they are active sites for both the generation of ROS and the recycling of these free radicals. Mitochondrial ROS are neutralized by the activity of the mitochondrial Mn^{2+} superoxide dismutase, which converts superoxide anion to hydrogen peroxide, which can therefore be transformed into water by glutathione peroxidase or converted to O_2 and H_2O by catalase. If not neutralized, mitochondrial ROS can damage pro-

teins, lipids, and nucleic acids. One key target of the damaging action of mitochondrial ROS is mtDNA. Mitochondria are unique as they posses their own maternally inherited mtDNA containing the genes for the 12S and 16S rRNAs and the 22 tRNAs required to translate the 13 mtDNA polypeptides that are key components of OXPHOS.

Several studies indicate that, with aging, accumulative mutations on mitochondrial mtDNA lead to impaired efficiency of OXPHOS. As a matter of fact mtDNA has a very high mutation rate, presumably due to the chronic exposure to ROS. Accumulation of age-related mutations can therefore be accompanied by the following cycling events:

- a decrease in oxidative phosphorylation and ATP synthesis capacity;
- an increase in mitochondrial ROS leakage as a result of their reduction in recycling through defective mitochondrial respiration;
- oxidative damage of mtDNA by mitochondrial ROS leading to an increase in the number of point mutations, expression of defective mitochondrial proteins, and further decline in OXPHOS activity [11].

Interestingly, these cyclic events seem to be amplified in post-mitotic cells where increased replication of mitochondria expressing deleterious mutations in their DNA has been found. This could be considered a compensatory response by which cells try to balance less efficient mitochondrial respiration with a greater number of mitochondria.

Mitochondria exert not only a critical role in maintaining cellular metabolism, but also control the release of pro-apoptotic factors such as cytochrome *c* (Cyt-*c*), apoptosis-inducing factor (AIF), SMAD/Diablo, endonuclease G, and Omi/HtrA2, all of which are normally contained within the organelle. Mitochondrial dysfunction is linked to the activation of the apoptotic machinery via the induction of the mitochondrial permeability transition pore (mPTP), a process that is set in motion by excessive mitochondrial Ca(II) (and Zn(II)) uptake or by oxidative stress. Thus, one can imagine a scenario in which aging induces a process that promotes an increase in the number of mtDNA mutations, higher ROS generation, and enhanced activation of the apoptotic machinery. When apoptotic cell death reaches a certain threshold level, tissue function starts to be compromised and disease symptoms begin. In that respect, tissues that have a higher reserve of cells able to undergo mitotic activity are better preserved from aging-related degeneration. Post-mitotic tissues like the brain cannot afford such a luxury making the whole system more vulnerable to aging.

3. Copper

Copper plays a basic role in all living systems being an active centre in proteins involved in the oxidase and oxygenase activities, electron transfer and controlling the level of oxygen radicals [12]. Copper can also play a key role in many diseases, including copper metabolism disorders, i.e. Menkes [13] and Wilson [14] diseases, neurodegenerative disorders like familial amyotrophic lateral sclerosis (FALS), Alzheimer's or prion diseases [15].

Cu becomes toxic in the case of excessive intracellular accumulation playing a role in initiating the generation of ROS and apoptotic processes. Cu is an essential trace element in all living organisms. The active centre of enzymes such as cytochrome *c* oxidase and Cu, Zn-superoxide dismutase where it cycles between the +1 and +2 oxidation states.

3.1. Copper homeostasis

Copper (and iron) is a redox-active metal that is both essential for the desired biological functionality and potentially harmful due to its role as a very effective catalyst for Fenton or Haber–Weiss type reactions producing the destructive hydroxyl radical and ending with serious oxidative damage. Cells have therefore developed a sophisticated machinery to ensure tight control of copper within the cell during their acquisition and distribution within the biological system.

On the other hand, the protein active centres need a particular metal ion, such that assembling of such centres driven by the protein donor system only is not sufficient to keep the protein activity unambiguous. To make this process unique the helper proteins (e.g. chaperones) are used.

Copper uptake into the eukaryotic cell is accomplished by the Ctr1 transporter which is very specific for Cu(I). Ctr1 is a integral membrane protein conserved from yeast to humans [16,17]. Ctr1 is present as a homo-trimer creating a pore between subunits [18,19] which matches the size and properties of Cu(I). Metalloreductases (Fre1 and Fre2) are needed to keep copper in the Cu(I) state, but how this oxidation state may be stabilized in the presence of oxygen before entering Ctr1 is not clear so far. Ctr1 has two attractive potential metal-binding regions comprising two methionine rich domains with MX_2M (extracellular N-terminal) motif and Cys-His rich (intracellular C-terminal) motif. The MX_2M motif has a strong impact on Cu(I) transport, while Cys-His rich domain function is still unknown. There is another Met motif (MX_3M) within the membrane located domain having a critical role in copper intake. Ctr1 is located mainly on the plasma membrane and in the intracellular vesicles and works as a Cu(I) transporter delivering the metal to cytosolic copper chaperones. It is very likely that copper has also Ctr1-independent low-affinity import system which may involve Cu(II) ions as well. Ctr2 [20], a transporter similar to Ctr1, was suggested to be responsible for Cu mobilization from vacuoles [21,22].

The delivery of metal ions to their specific pathways into the cell is mediated by metallochaperones that function to protect metal from intracellular scavengers and provide metal directly to target proteins. The chaperones known for the copper ion are generally divided into three functional classes reflecting whether delivery is assisted to the trans-Golgi network (Atx1), to superoxide dismutase (CCS) or to mitochondrial cytochrome *c* oxidase (Cox17) (Fig. 1).

The yeast Atx1, the bacterial CopZ and the human homologue (known as HAH1 or Atox1) plays the same role in delivering copper to an intracellular copper transporter in the Golgi and further into copper enzymes [23–25]. Atx1 is a cytosolic metallochaperone

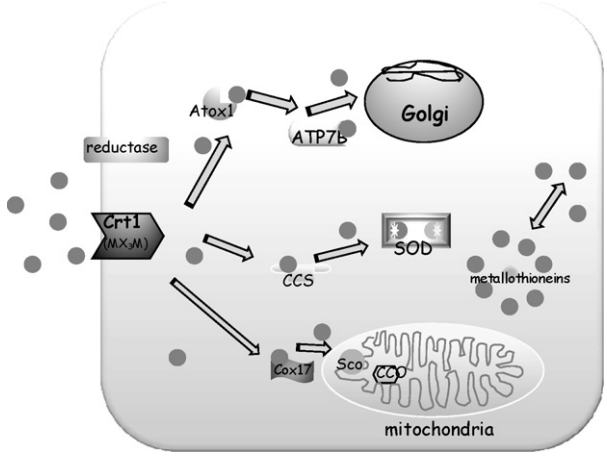

Fig. 1. Scheme of copper(I) trafficking pathways in mammals.

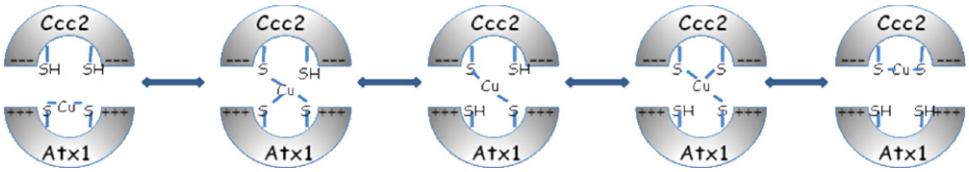

Fig. 2. Proposed mechanism for yeast model of copper transfer from Atx1 to Ccc2.

shuttling copper ions to the copper transporter Ccc2 and further into the secretory pathway to the multicopper oxidase Fet3. Atox1 (HAH1) delivers Cu(I) to the Menkes and Wilson ATPases (ATP7A and ATP7B) for ultimate incorporation into ceruloplasmin. It is proposed that Atox1 can transfer metal ion via direct interaction with homologous domains of the P-type ATPases. Any disruption in this copper trafficking pathways can lead to Wilson or Menkes disease [26].

Many structural studies on copper Atx1 and Atx1-like metallochaperones show that these proteins are composed of a specific βαββαβ fold with a similar tertiary structure and the metal-binding site exposed at the protein surface [24,25,27,28]. It is also well documented that the Atx1 and its homologues are able to bind single metal ion via two cysteine residues from the conserved MT/HCXXC motif which is also present in the Ccc2 (in two domains) and in the Wilson and Menkes proteins (in six domains). These metal-binding sites in chaperones are very flexible and have the same fold as their protein partners, Ccc2 and ATPase [29,30]. Relative to the stable complex with five or six chelate ring size, Atx1 and Atx1-like metallochaperones with copper ion form a 15-member chelate ring which are relatively stable because of side chain orientation, hydrogen bonding, hydrophobic and electrostatic interactions. Differences in the metal binding in this class of chaperones [30], are associated with the variation in length of the first helix constraining the position of metal-binding Cys, which may determine the coordination preferences of Cu(I) ion. After metal binding, the metallochaperone undergoes a conformational change that allows further metal transfer into its partner. Stabilization of the protein complex, in the chaperones of eukaryotes, is driven by the turn of the N-terminus of helix 1 and the positive charge of the Lys residue stabilizing the metal-binding site [27,29]. This Lys is proposed to play a functional role also in modulating the copper transfer process [24,29]. An arginine and a number of lysine residues conserved in Atx1 and its homologues generate a positively charged surface and interact with the negatively charged residues on the surface of protein partner like in the protein–protein docking [31]. On the basis of the spectroscopic studies of Atx1 it is proposed that the mechanism of copper transfer to the partner implies the formation of two- and three-coordinate intermediates involving Cys residues from both proteins (Fig. 2) [32,33]. Other conserved residues localized near the metal-binding site like threonine or methionine probably assist in the copper transfer by partially neutralizing the overall negative charge on the coordinate intermediate and modulate interactions of metallochaperone with Ccc2 or ATPases through hydrogen bonding (Thr) and affecting the loop structure (Met) [20,30,33].

Physiological delivery of copper into eukaryotic SOD1 enzyme in post-translational metallation process requires the specific CCS metallochaperone and occurs via direct protein–protein interaction involving a CCS–SOD1 heterodimeric intermediate (Fig. 3) [34,35].

CCS co-locates with SOD1 in many mammalian tissues, including brain and the motor neurons in the spinal cord in patients with amylotropic lateral sclerosis, pyramidal neurons in the cortex and cerebellar Purkinja cells but CCS levels are not strictly correlated with SOD1 levels [36–40]. CCS is present in the cytoplasm and in the mitochondrial IMS [41,42]. CCS is a 30-kDa protein having three structurally conserved distinct domains that carry out the functions of copper binding, copper transfer and docking into the SOD1 target. Domain I exhibits homology to an Atx1-like sequence with metal-binding motif, MX/CXXC at the N-terminus; domain II is homologous to SOD1 (the sequence identity between human CCS and SOD1 is close to 50%); domain III is the most conserved unique sequence encompassing CXC motif responsible for copper binding and SOD1 metallation [43–46]. Spectroscopic data strongly suggest

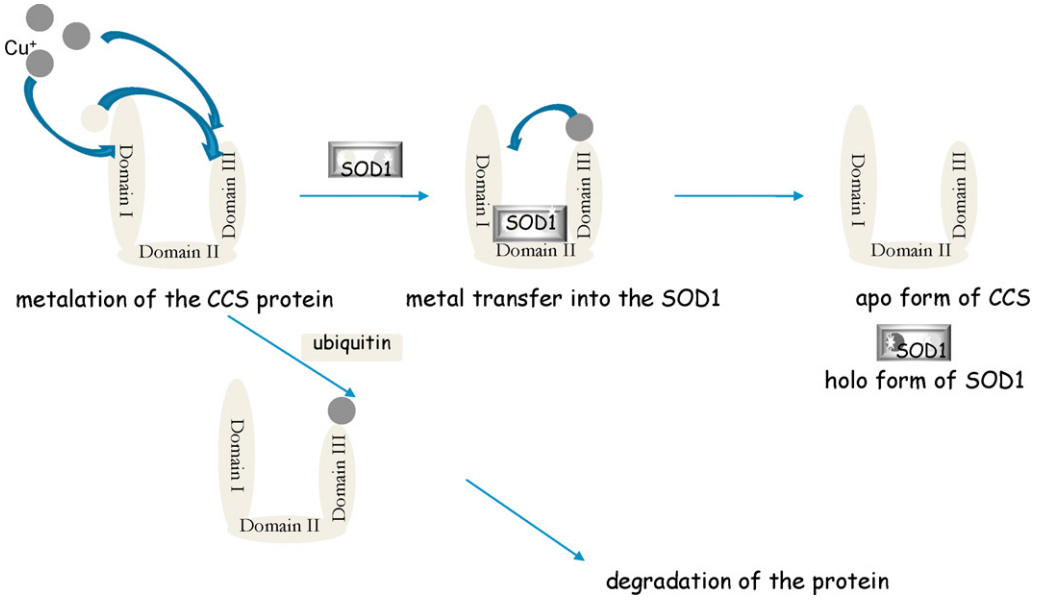

Fig. 3. Schematic model of the copper transfer in the presence of metal abundance.

that cysteine residues from domain I (CXXC) and domain III (CXC) adopt a proximal orientation and both are involved in copper binding [47,48]. However, metallochaperone lacking first domain can still insert copper into SOD1 *in vivo* [43]. In yeast under conditions of excess copper, domain I is not required for copper delivery to SOD1 [48]. The situation seems to be different for mammalian cells where domain I is required for the activation of SOD1 [48,44]. Most probably when the level of copper increases the conformational change upon metal binding to the CXC motif result ubiquitination of CCS and degradation of the protein (Fig. 3) [49]. Unlike to the first and third domain, domain II is responsible for the interactions with SOD1 [50] and facilitates metal transfer from CCS to SOD1. The crystal structure shows that this domain contains two loops comparable to zinc sub-loop and electrostatic loop of SOD1 [51]. In spite of the sequence and structural similarity to superoxide dismutase CCS is catalytically inactive most probably because of the presence an aspartate residue (D201) instead of one of the copper-binding histidines (H120) in SOD1 [39,52]. Cu is transferred from third domain of metallochaperone to four histidine ligands of Cu/Zn SOD in an oxygen-dependent mechanism by the formation of disulfide bridge between CCS and SOD1 and series of ligand reactions [39,40,53,54]. In addition to CCS role in copper delivery, it also takes part in the catalytic formation of an essential disulfide bond in SOD1 [39]. Copper ion can be also loaded into SOD1 in the absence of metallochaperone in a CCS-independent mechanism. This process occurs in the presence of reduced form of glutathione (GSH) [55]. However, it is noticed that in the presence of CCS there is no significant dependence on reduced GSH levels for Cu loading onto Cu/Zn SOD [5,47]. Moreover when all SOD1 is metallated copper binding to the monomer of CCS might also induce a conformational change and trigger CCS ubiquitination and proteasomal degradation probably as the result of exposition of Lys256 on the surface of domain III [38].

In respect to the amount required for activation of mitochondrial cytochrome oxidase, the level of copper in the mitochondrial matrix is by an order of magnitude higher [56]. Copper insertion into cytochrome oxidase requires a number of accessory proteins including Cox11, Cox17, Cox19, Cox23, Sco1 and Sco2 [57]. Cox17 is a metallochaperone acting as a donor of Cu(I) to Sco1 and Cox11 [58]. Cox17, originally identified in yeast, is a small cysteine rich protein that, depending on its redox state, is able to coordinate from one to four copper(I) ions [25,59]. Cox17 is present in both, cytosol and intermembrane space (IMS) of mitochondria. Import of yeast Cox17 into the IMS is catalyzed by the disulfide system of Mia40 and Erv1 proteins. Mia40 and Erv1 favour the formation of the partially oxidized state ($Cox17_{2S-S}$) which is the active form of metallochaperone responsible for copper transfer within the IMS [60]. Human Cox17 is a key protein supplying copper ions to cytochrome *c* oxidase with the involvement of other proteins like Sco1, Sco2 and Cox11 [49].

In comparison with Atx1, the global fold of Cox17 is quite unusual with a CHCH motif of about 40 residues and a large unstructured segment which is completely unrelated to its protein partners, Sco1 [61] and Cox11 [62]. Copper(I) bound to the Cox17 makes the protein more structured and less flexible. Three of the six strongly conserved Cys residues form a CCXC motif involved in copper binding and are important for Cox17 function. In the yeast Cox17, the metal ion is digonally coordinated by the first and last Cys of the CCXC motif. This binding requires isomerisation of the disulfide from Cys26/Cys57 to Cys24/Cys57 [51]. Human Cox17 binds Cu(I) in a different manner, via sulfur atoms of two adjacent Cys in CCXC. Copper coordination determines local structural rearrangements with formation of a turn with two consecutive Cys close to each other in an optimal conformation for Cu(I) binding [28]. Similarly to Atx1, Lys20 can contribute to the stabilization of the overall negative charge resulting from binding of Cu(I) to two cysteinate thiols. Furthermore, hydrophobic contacts between residues located at the end of the C-terminal helix and at the N-terminus direct the metal-binding site toward the protein surface and expose it to the solvent, favouring copper transfer to the protein partners of Cox17 [28]. Metal transfer to Sco1 can be driven by the higher number of metal-binding ligands (in Sco1 copper ion is coordinated by two cysteines and one histidine) [53].

Ccc2 in *S. cerevisie* or human homologues Menkes and Wilson disease proteins [13,14] (ATP7A and ATP7B) copper transporting ATPases, deliver copper to multicopper oxidase Fet3 [63,64] or, in the case of humans ceruloplasmin. Both proteins are critical for copper uptake. ATP7A is present in all tissues except liver, while ATP7B is expressed primarily in the liver (Fig. 4). Both transporters can deliver copper to the multicopper oxidase Fet3p in yeast. Mutations in Wilson disease protein, which, among its functions, mediates secretion of excess copper into the bile may causes copper overload in the liver, neurons and other tissues. Mutations in ATP7A that also transports copper through basolateral membrane, results into copper deficiency (Menkes syndrome).

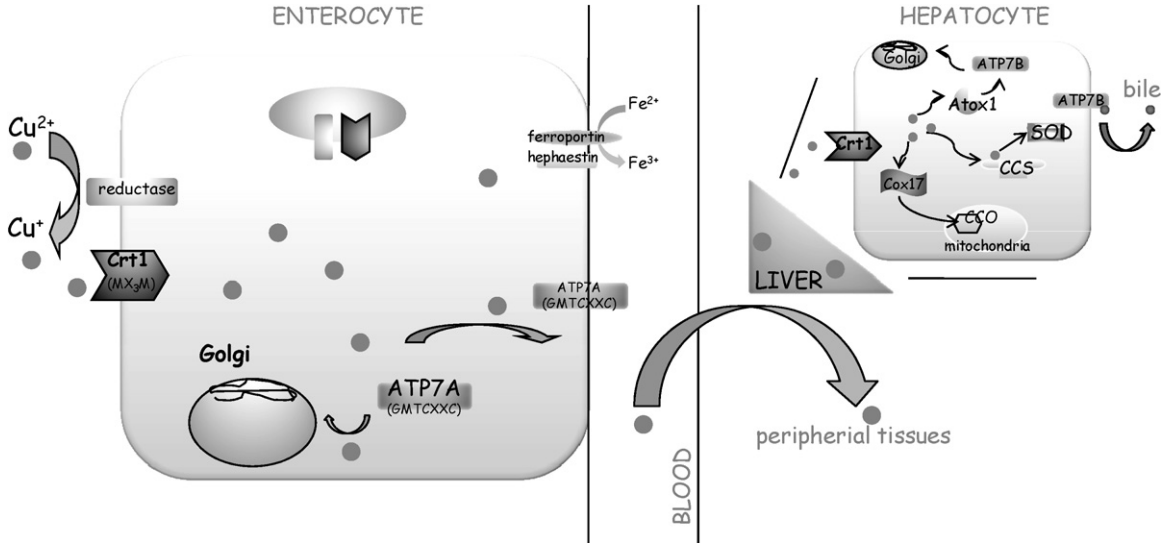

Fig. 4. Schematic model of intestinal copper absorption and its peripheral distribution.

A characteristic feature of P-type ATPases is the presence of multiple Atx1-like metal-binding domains in the N-terminus. As in the case of copper chaperones, the metal-binding motif consists of the sequence XX′CX″X‴C. This highly conserved residue is not directly involved in the metal ion binding but rather takes place in hydrophobic interactions stabilizing the folding of the metal-binding loop. X″ position is highly variable, while in the case of eukaryotic ATPases X″ is Ser or Ala [65].

The transfer of copper from the chaperone to ATPase and then to secretory compartments is not well understood. Cu is transferred from the surface of Atox1 chaperone to a metal-binding domain in the N-terminus of the metal transporting ATPases ATP7A and ATP7B via specific protein–protein interactions [66]. How copper is transferred from the N-terminus site of the transporter to the transmembrane domain to be moved across the membrane is not yet known. How then copper is delivered and inserted into the protein (ceruloplasmin) is also not well understood [67].

3.2. The copper metabolism protein MURR1/COMMD1

When dealing with the pathophysiology, genetics and the treatment of human diseases, naturally occurring canine genetic diseases have provided useful models [68], due to the close evolutionary relationship and higher degree of DNA sequence identity. Copper is one of the essential heavy metals in man's life that is required to be kept under rigorous homeostatic control [69]. As just mentioned, two copper transport ATPases have been identified in man and rodents which, when dysfunctional, cause either copper deficiency (Menkes disease) or copper accumulation in various tissues (Wilson disease). An autosomal recessive copper toxicosis has been also described in Bedlington terriers [70,71], and characterized by the accumulation of copper in the liver. The copper toxicosis locus has been localized to canine chromosome region CFA10q [26,72] a region homologous to human chromosome region HSA2p13–21, corresponding to *ca.* 9 Mb [73]. The gene was further identified as a gene in close proximity to the imprinted murine gene U2af1-rs1 [74], and it was initially designated as *MURR1* (Mouse U2af1-rs1 region 1). A genomic deletion mutation of exon 2 of the *MURR1* gene is deleted in both alleles of all affected Bedlington terriers [75], resulting in the complete absence of this protein probably due to protein instability. This implies that *MURR1* plays a major role in copper metabolism. Furthermore, a new family of proteins in diverse species, including flies and yeast, that have structural and functional homology to *MURR1*, has been discovered [76]. Thus, a new nomenclature for this family of proteins has been assigned as *COMM* (copper metabolism gene *MURR1*) domain or *COMMD*, which at present consists of 10 subgroups (*COMMD1–COMMD10*), where *MURR1* is now known as *COMMD1* [67]. These proteins are defined by the presence of a conserved and unique motif that is leucine-rich, 70–85 amino acid long, and near the carboxyl terminus, which functions as an interface for protein–protein interactions (Fig. 5).

COMMD1, ubiquitously expressed in tissues and cell types [78], mainly occurs in the cytoplasm. The actual function of *COMMD1* is not yet clear, but interacts specifically with *ATP7B* (the Wilson disease protein), the P-type ATPase involved in the transport of copper in the liver [79], but not with other copper-binding proteins, such as *ATP7A* (the Menkes disease protein), *ATOX1*, *SOD*, or *CCS*. The failure to detect any interaction with the Menkes disease protein supports the concept of a specific functional interaction in hepatocytes and is consistent with the liver-specific phenotype of impaired copper homeostasis observed in affected Bedlington terriers. The interaction with *COMMD1*, which does not require the carboxyl terminus of *ATP7B* residing in the cytoplasm [70], was shown to be mediated via the copper binding, amino terminus of this ATPase, in a way that resembles the interaction between *ATP7B* and the cytosolic copper chaperone *ATOX1* [80]. N-terminal regions of *ATP7B* and *ATP7A* contain six metal-binding domains, each *ca.* 70 amino acids in length and containing one MXCXXC motif. Despite their sequence and structural similarity, clearly there are sufficient differences in sequence and/or structure that confer specificity of interactions with proteins such as *COMMD1* and differences in their trafficking pathways.

The screening of a human liver cDNA library using the N-terminal region of *ATP7B* as a bait to search for interacting partners identified the protein p62 (dynactin 4; DCTN4) [81], a subunit of dynactin, as potentially interacting with *ATP7B*. The p62 subunit of dynactin was shown to interact with the N terminus of *ATP7B* but not *ATP7A*. The interaction was copper-dependent and required the CXXC motifs and the region between metal-binding domains 4 and 6. Upon copper binding to the N-terminal metal-binding domains of the ATPases, a conformational change occurs which exposes a specific targeting signal within the N terminus, which enables the protein to traffic through interaction with components of the cell sorting machinery [82]. A model was therefore

(a)

Orthologs in other Species

Conservation (% against human sequence)

	Mammals	*Fish*	*Dictyostelium*	*Unicellular protozoa*
COMMD1	92-95	72-76	58	-
COMMD2	97-99	88	67	50
COMMD3	93	71	53	52
COMMD4	94-97	79	60	50
COMMD5	89-93	70	52	55
COMMD6	93	88	76	-
COMMD7	94-95	81-82	62	47
COMMD8	86-92	70	58	-
COMMD9	93-95	77	68	55
COMMD10	93-99	76-81	56	48

(b)

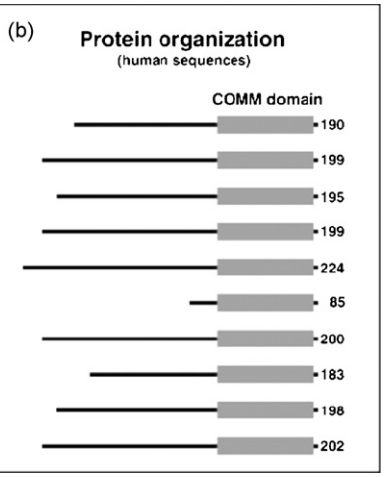

Fig. 5. (a) Phylogenetic conservation of the COMMD gene family. Orthologs for the human COMMD genes were identified in various mammalian and fish species and their level of conservation compared against the corresponding human protein. In addition, orthologs could be identified in *Dictyostelium discoideum*. Finally, COMMD orthologs are also present in unicellular protozoa such as *Trichomonas vaginalis*, *Entamoeba histolytica*, and *Tetrahymena thermophila*. (b) Schematic representation of the COMMD family of proteins. The conserved COMM domain is shown in grey, along with the respective amino acid length of each protein in humans. (Reprinted with permission from Ref. [77].)

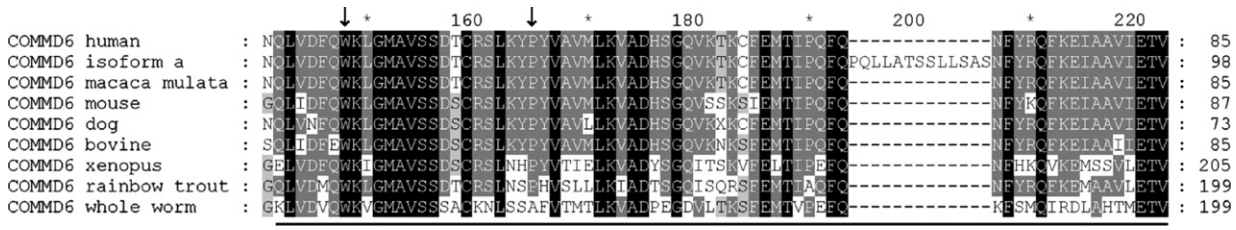

Fig. 6. Alignment of the COMM domain of COMMD6 of different species. Black, dark-grey or light-grey background colours indicate a 100%, 50% or 20% degree of conservation respectively. (Modified from Ref. [88], reprinted with permission.)

suggested for copper-induced conformational changes and subsequent microtubule-mediated apical trafficking of *ATP7B* in response to elevated copper concentrations [72,83]:

- when intracellular copper levels are low, the metal-binding domains of *ATP7B* are not entirely saturated with copper, and the protein is predominantly located at the TGN;
- when copper levels become elevated, all available domains become loaded with copper;
- the fully loaded N-terminal region undergoes a conformational change, and the protein traffics to the basolateral membrane;
- here p62 interacts with the N terminus of *ATP7B*, in a mechanism that may involve the N-terminal 63 amino acids, to target the copper-bound protein for microtubule-mediated trafficking toward the biliary canalicular membrane.

This model may explain why p62 does not interact with the similar copper transporter *ATP7A*. The N termini of the two ATPases differ significantly in the region between binding domains 4 and 6 and within the region prior to domain 1, which comprises only 10 amino acids in *ATP7A* and 63 in *ATP7B*. Copper-loaded *ATP7A* would traffic to the basolateral membrane but would not be recognized by p62 for microtubule-mediated apical trafficking. The interaction with *COMMD1* can involve the same region but fully loaded *ATP7A* may not recognize *COMMD1*.

COMMD1 interacts with the human epithelial sodium channel, a key regulator of sodium movement across epithelia, and to inhibit its activity [84]. *COMMD1* has been further reported to inhibit the degradation of *IκBα*, thus maintaining the transcription factor NF-κB in an inactive form [85]. Moreover, it was demonstrated that *COMMD1* regulates the nuclear function of NF-κB by affecting its association with chromatin [67].

An inverse correlation between the *COMMD1* protein and the intracellular level of copper was shown on human embryonic kidney 293 cells [86], and a negative regulator of *COMMD1* was identified as a protein called XIAP (X-linked inhibitor of apoptosis), a potent suppressor of apoptosis [77]. XIAP decreases the level of *COMMD1* by promoting its ubiquitination and degradation. As a matter of fact, XIAP binds copper, which determines a reversible conformational change and enhances its degradation, thus lowering the apoptotic threshold [87].

As already mentioned, a screen to identify additional *COMMD1*-binding partners led to the identification of nine proteins with homology to *COMMD1* [67]. These proteins, designated *COMMD2–COMMD10*, share a highly conserved 70–85 residue C-terminal domain, the *COMM*-domain (Fig. 6). The N-terminal regions of the 10 *COMMD* proteins vary in sequence and in length, ranging from 18 residues for *COMMD6* to 151 residues for *COMMD5* (Fig. 5). All 10 *COMMD* proteins interact with each other, and the C-terminal *COMM* domain serves as an interface for these *COMMD–COMMD* interactions [67,88].

The full-length *COMMD1* protein (MW 21 kDa) is prone to aggregation: since the C-terminal *COMM*-domain is involved in *COMMD–COMMD* protein interactions [67], this domain is likely to aggregate at high concentrations. In order to obtain structural information, three soluble constructs for the N-terminal domain encompassing the regions 1–108, 1–115, and 1–118 were generated [89]. The variant 1–108 exhibited the best soluble expression profile and was folded with a high α-helical content, and was a single, monomeric species (apparent MW 19 kDa) [89]. As determined by NMR, the N-terminal domain adopts a compact, monomeric, and completely α-helical fold in the absence of the C-terminal COMM-domain (Fig. 7). The solvent-exposed surface area is very high and almost all the hydrophobic residues are sequestered within the protein interior. At the N terminus, an irregular helix (α1) and a short helix (α2) are connected via a long disordered loop (residues 20–29).

The alpha-helical structure found, does not resemble to any other helical protein and its compact nature suggests that full-length *COMMD* proteins are modular, with specific functional properties for each domain.

In order to assess which part of the *COMM* domain (residues 116–185) of *COMMD1* is involved in protein–protein interactions, two truncated constructs, *COMMD(61–154)*, the product of exon 2, and *COMMDΔ(61–154)*, the product of exons 1 and 3, were investigated [90]. Because each of the two peptides was able to form dimers, the interface for protein–protein interactions is likely to lie

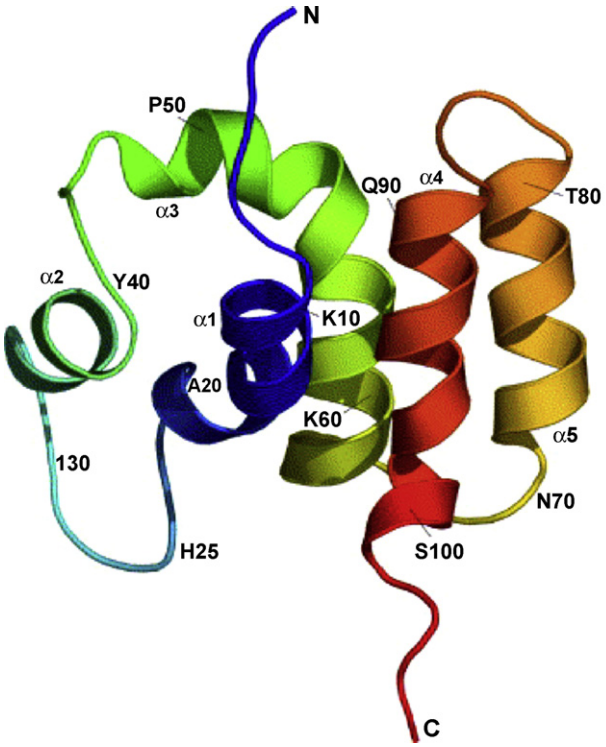

Fig. 7. Ribbon diagram of the averaged and energy minimized structure of the 1–108 region of *COMMD1*. The N-terminus is coloured in blue and the C-terminus in red. (Reprinted with permission from Ref. [89].)

at the junction between exons 2 and 3. In the same report, various experimental data including native-PAGE, EPR, UV–vis electronic absorption, intrinsic fluorescence spectroscopies as well as DEPC modification of histidines, demonstrated that *COMMD1* specifically binds 1.0 equiv. of Cu(II) (K_d *ca.* 3–5 mM) and does not bind other divalent metals. The exon 2 product, *COMMD(61–154)*, was also able to bind Cu(II) as well as the wild type protein. These last observations are relevant at the light that the product of exon 2 is deleted in dogs affected by copper toxicosis. The protection of DEPC modification of *COMMD1* by Cu(II) provided evidence of His101 and His134 being involved in metal binding; whereas the involvement of Met110 was demonstrated by fluorescence studies.

In conclusion, COMMD proteins represent a set of regulatory factors involved in several biological processes important for cellular homeostasis. These include the regulation of copper and sodium transport, NF-κB activity, cell cycle progression, and others that have yet to be determined. One possible unifying mechanism of action for the regulatory properties of the COMMD protein family might be their involvement in the ubiquitin–proteasome pathway. As was recently described, COMMD1 facilitates the termination of the NF-κB response through its association with a Cul2-containing ubiquitin ligase [91]. Given that COMMD1 can also interact with other Cullin proteins, COMMD proteins are likely to associate with other ubiquitin ligases. The characterization of these enzymes and their relevant substrates will prove to be important in uncovering additional pathways that are controlled by COMMD proteins.

3.3. Metallothioneins

In recent years the importance of cellular interactions between neurons and glial cells (especially astrocytes) has been established in normal as well as in disease states. A great piece of information has been gained by investigating a family of small cysteine rich proteins called metallothioneins (MTs), primarily produced by astrocytes. The absence of MTs has been shown to result in impaired recovery from CNS (central nervous system) trauma, while overexpression yields improved recovery. Such neuroprotective functions have been attributed to the free radical scavenging and heavy metal-binding properties of MTs. It is however emerging that an extracellular activity of MTs may also be important since exogenous MT has been shown to interact directly with neurons to promote neuronal survival, neurite outgrowth and axonal regeneration, thus raising the possibility that MT might be released from astrocytes following CNS injury.

There are four mammalian MT isoforms, MT-1 to MT-4. MTs are small (61–68 amino acids) proteins, and have a tertiary structure composed of two distinct domains, the α- and β-domains (Fig. 8) [92]. MT-1 and MT-2 share very high sequence homology and similar expression profiles and are the most abundant isoforms within the brain, found primarily in astrocytes. MT-3 is found mainly within the brain, and at much lower levels in some peripheral tissues. MT-4 isoform is not found in the brain at all. The role of MTs in the brain is unclear, although it is generally agreed that they play relevant roles in free radical scavenging and controlling the availability of zinc, possibly in a redox-dependent fashion [93]. MTs can bind a range of heavy metals including copper, zinc, cadmium and others [94]. Many of these metals can also be found coordinated to MT *in vivo* following administration of the metal ion to the organism. MT-1/-2 expression is increased in prototypical neurodegenerative diseases [84,95]. MT-1/-2 are stress proteins that can be induced *in vivo* by many factors, including zinc [96].

In its fully metallated form, mammalian MT adopts a conformation with distinct metal–thiolate clusters located within two domains with stoichiometries of $M_3(S_{cys})_9$ (in the N-terminal β domain) and $M_4(S_{cys})_{11}$ (in the C-terminal α domain) for divalent metal ions [98–100]. The folding of the protein chain around the clusters is entirely driven by the coordination of the metal ions. The exact mechanism of metal ion chelation (i.e. the order of metal binding, the eventual cooperativity of the process, the rate of binding of each metal ion) is not well clarified so far, but this information is probably the key to unravelling the role of MTs in the cell.

The metal-free protein, referred to as apo-MT or thionein (T), was early reported to be very unstable and very rapidly degraded *in vitro* [101]. Until recently, it has been generally agreed that the structure of apo-MT is an oxidatively unstable random coil devoid of any function.

It was not known until 2001 that apo-MT could be directly detected in the liver, kidney and brain of rats [102], in quantities almost equal to that of metallated MT and also with greater stabil-

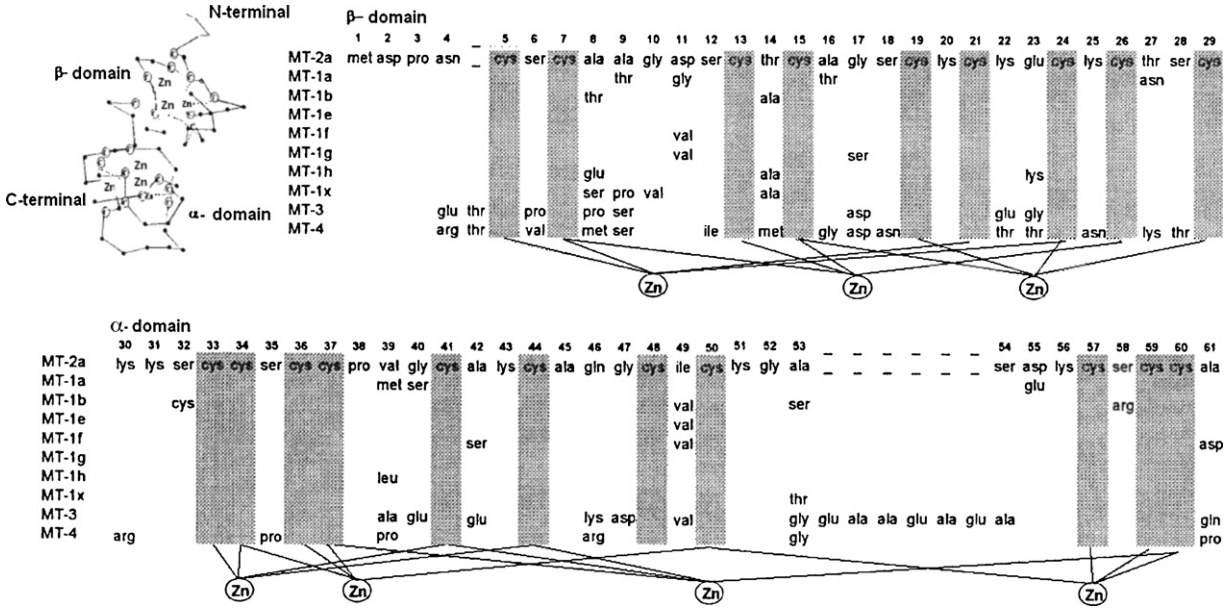

Fig. 8. Schematic representation of the MT structure together with amino acid sequences for human MTs. Shown are the α and β-domains with the conserved position of the cys residues highlighted in grey. Amino acid residues differing from MT-2a are given. Metal coordination positions are based upon those derived from rat MT-2. (Reprinted with permission from Ref. [97].)

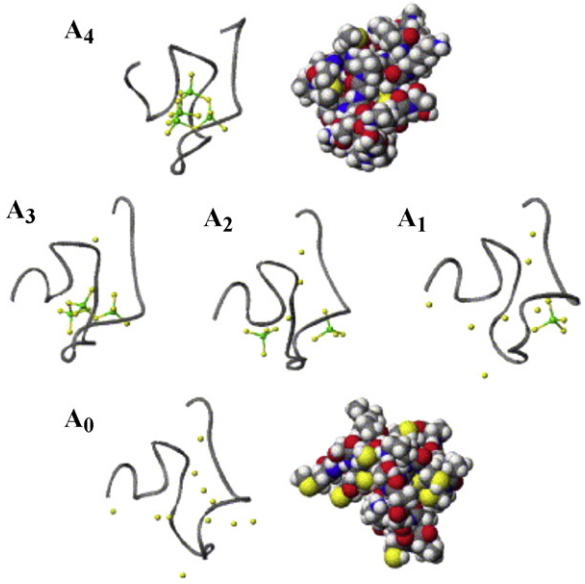

Fig. 9. Ribbon and ball and stick representations of the energy-minimized structures of the α domain at each step of the sequential demetallation, where the ribbon represents the polypeptide backbone and the green and yellow spheres represent the Cd^{2+} ions and the cysteinyl sulfurs, respectively: (A_4) Cd_4α-rhMT (A_3) Cd_3α-rhMT (A_2) Cd_2α-rhMT (A_1) Cd_1α-rhMT (A_0) apo-α-rhMT. Beside A_4 and A_0 are space-filling representations of Cd_4α-rhMT and apo-α-rhMT, respectively. Atom legend: Grey, C; Blue, N; Red, O; Yellow, S; Green, Cd. Similar results were obtained for the β domain. (Reprinted with permission from Ref. [105].)

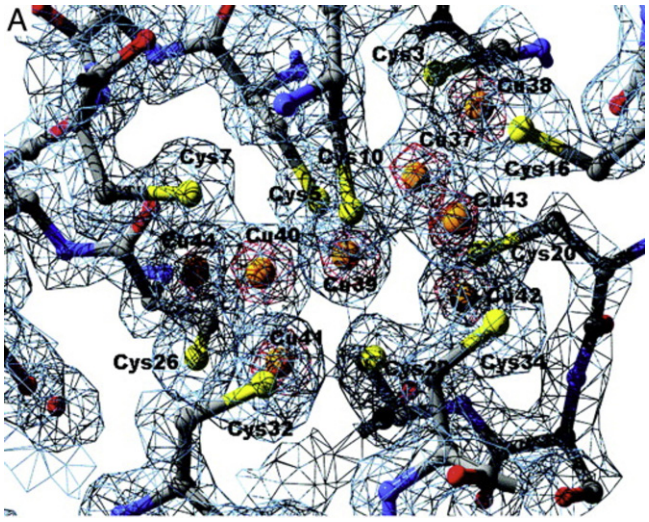

Fig. 10. Different aspects of copper binding in Cu_8-MT. (A) Close-up of the electron density in the metal-binding region showing the 10 Cys residues and the 8 coppers bound to them. 1σ contoured $2F_o–F_c$ map is shown for the Cys residues and the coppers (cyan) and 17σ contoured anomalous difference Fourier map for the coppers (red). (Reprinted with permission from Ref. [115]; Copyright (2005) National Academy of Sciences, U.S.A.)

ity *in vivo*. This was the first report of the existence of apo-MT in tissues under normal physiological conditions.

Metal-free MT can be easily generated *in vitro* at low pH. Computational modeling techniques were used to probe the behavior of apo-MT [103], and also, the partially metallated and fully metallated states [93,104]. All forms of the protein showed retention of a significant degree of structural features upon demetallation to generate the metal-free protein (Fig. 9).

Interestingly, an increasing number of H-bonds upon demetallation, is probably stabilizing the domains of apo-MT. It follows that the metal ions are likely to act as a structural template for the polypeptide backbone. Moreover, the cysteinyl sulfur atoms rotate from the interior to the exterior of the domain, thus increasing the probability of metallation. The predicted retention of conformation upon demetallation has been experimentally verified [106].

The maximum number of metal ions bound by the α and β domains of MT is determined by the coordination properties of the metal ion. Four Cd(II) and Zn(II) are bound in the α domain and three in the β domain, while, in the case of Cu(I), Ag(I) and Hg(II), both the α and β domains can achieve domain occupancies of four, six or nine metal ions.

As for the copper-MT (Cu-MT), its exact role has been elusive so far, but it has been strongly supported that it may act as depository for copper transfer into copper apo-proteins [107,108], and/or copper apo-chaperones [109,110], involved in copper trafficking. Moreover a role in copper detoxification has been shown in yeast and mammals [111,112].

Cu-MT contains 53 amino acids, of which 12 are cysteines, and 6–8 Cu(I) ions. Solution structures of increasing accuracy were provided by NMR [113], but without structural details of the copper cluster. A model for the copper cluster was suggested by the Ag_7-MT derivative [114]. In the latter model, 10 cysteines were involved in the metal coordination.

The yeast Cu-MT was finally crystallized and its structure determined at 1.44-Å resolution [115]. The structure is characterized by a Cu(I) thiolate cluster consisting of six trigonally and two digonally coordinated Cu(I) ions (Fig. 10). The protein backbone is random coil with the loops wrapping the copper cluster, located in a cleft where it is bound to 10 cysteine residues. The structure may be consistent with Cu-MT playing an active role in the delivery of copper to metal-free chaperones.

The homeostasis of zinc and copper is tightly regulated and essential for brain physiology [116]. Dysregulated metabolism occurs in most neurodegenerative disorders. As already stated, MT-3, also known as the growth inhibitory factor, is a small non-inducible cysteine- and metal-rich protein mainly expressed in the brain. Like other mammalian metallothioneins MTs, MT-3 binds with a high affinity Cu(I) and Zn(II), and Zn_7MT-3 has been suggested to actively participate in synaptic cycle of zinc vesicles [117]. MT-3 also occurs in the extracellular space. MT-3 is isolated as a $Cu(I)_4$,$Zn_{3–4}$MT-3 species. This feature, joined with its down-regulation in Alzheimer's affected humans [118], makes MT-3 an interesting candidate for a major role in zinc and copper metabolism in the brain.

MT-3 binds Zn(II) through the array of 20 conserved cysteines into two metal–thiolate clusters, i.e. $Zn_3(CysS)_9$ and $Zn_4(CysS)_{11}$. Both native $Cu(I)_4$,$Zn_{3–4}$MT-3 from human and bovine brains and the *in vitro* prepared $Cu(I)_4$,Zn_4MT-3 revealed a $Cu(I)_4$-thiolate cluster in the N-terminal β-domain and a $Zn(II)_4$-thiolate cluster in the C-terminal α-domain [119,120], with digonal and trigonal Cu(I) coordination. Sulfur oxidation may represent the source of reducing equivalents for dealing with extracellular Cu(II) with successive binding of Cu(I) to the protein. As a matter of fact, Zn_7MT-3 could be shown to act as a scavenger for free Cu(II) ions through their reduction to Cu(I) and binding to the protein [121]. During the reaction cysteinate ligands are oxidized to disulfides with concomitant release of Zn(II). As a consequence Zn_7MT-3, in the presence of ascorbate, could act as a scavenger for the copper-catalyzed hydroxyl radical production [110].

3.4. Toxicity of Cu and its role in apoptosis

If the delicate balance between the uptake and efflux of Cu ions does not function correctly, Cu can become cytotoxic and contribute to the development of several pathologies such as cancer, diseases of the nervous system, and aging [122]. Human genetic

disorders such as X-linked Menkes disease and autosomal recessive Wilson's disease also highlight the importance of intact cellular transport and accumulation mechanisms. For instance, Wilson's disease (hepatolenticular degeneration) is an inherited autosomal recessive disorder of Cu transport characterized by the failure to incorporate Cu into ceruloplasmin in the liver, and the failure to excrete Cu from the liver into bile. In the Long–Evans Cinnamon (LEC) rat, a mutant animal model of Wilson's disease [123,124], Cu accumulation could play a role in initiating the generation of ROS and apoptotic processes [125,126]. In addition to the generation of ROS, Cu may manifest its toxicity by displacing other metal cofactors from their natural ligands.

The copper that participates in producing ROS is called "free" copper, probably a somewhat inappropriate term, that refers to copper more loosely bound to proteins and other molecules. About 90% of blood copper in humans is covalently bound to ceruloplasmin, while the remaining 10% is loosely bound to albumin and other molecules. In all cells, there is a considerable pool of free copper, some of it stored in metallothionein. This amount of free copper produces ROS throughout life, and it is only with aging or under neurodegenerative conditions that impaired protection by antioxidant defence mechanisms yields imbalance and oxidative stress.

The age-related increase in oxidative brain damage is best exemplified by products of the lipid peroxidation [127], protein oxidation [128], and oxidative modifications in nuclear and mitochondrial DNA [129]. An increase in protein carbonyl levels has been demonstrated for various brain regions including the hippocampus [130].

Age-related memory impairment is correlated with a decrease in brain and plasma antioxidants [131], including the intracellular glutathione concentration and the ratio glutathione/glutathione disulfide in all mammalian brain regions tested including the hippocampus.

3.5. Copper in neurodegeneration

Various types of oxidative damage have been noted in Alzheimer's disease (AD), including glycation, protein oxidation, lipid peroxidation, and nucleic acid oxidation [132]. An Aβ-amyloid-42-centred model has been developed [133], suggesting that the amyloid peptide induces and antioxidants inhibit protein oxidation, lipid peroxidation, ROS production, and many other markers of oxidative stress. In the same way, studies in human post-mortem material indicate that ROS are important in the pathogenesis of sporadic Parkinson's disease (PD) [134]. There are also consistent observations of the impaired functioning of mitochondrial complex I, with consequent aggregation and accumulation of α-synuclein [135].

Besides the production of ROS, a role for copper, as well as for other metals, such as iron, manganese, and the redox-silent zinc, in neurodegeneration emerges from the direct binding at specific sites in the involved proteins. Only SOD1 is a cupro-enzyme, but the amyloid precursor protein and its Aβ fragments, the prion protein and α-synuclein all possess sequence motifs that offer specific binding sites to Cu(II) ions [6,15]. As a matter of fact, several experimental evidence connect copper to AD. In fact the aggregation of Aβ is promoted by copper and its neurotoxicity depends on catalytically generated H_2O_2 by Aβ-copper complexes *in vitro*. Moreover, the characterization of Cu(II) interacting with α-synuclein demonstrates that this metal is effective in accelerating protein aggregation at physiologically relevant concentrations without altering the resultant fibrillar structures [6]. Once bound to its target sequence, metal-catalyzed oxidation (MCO) of proteins implies reduction of Cu(II) by a suitable electron donor such as NADH, NADPH, ascorbate or mercaptans. Cu(I) ions bound to specific metal-binding sites on proteins react with hydrogen peroxide to generate hydroxyl radical [136], which immediately oxidizes neighbouring amino acid residues. MCO of proteins is a highly selective reaction that occurs primarily at protein sites with transition metal-binding capacity and is mainly a site-specific process in which amino acids at metal-binding sites on the protein are preferentially oxidized [137]. The ROS formed in MCO systems can lead to oxidation of amino acid residue side chains, formation of protein–protein cross-linkages, and oxidation of the protein backbone resulting in protein fragmentation.

A third possible link between copper homeostasis, oxidative stress and neurodegeneration is emerging from the view that mitochondrial dysfunction may be the shared basis for pathogenesis of all these diseases [123]. In fact, many of the implicated proteins have direct physical involvement with mitochondria or mitochondrial proteins [123], and it is likely that such interactions are modulated by copper, as well as by other redox-active or redox-silent metal ions. Few examples are provided. In transgenic mice overexpressing the amyloid precursor protein, the mitochondrial protein importation machinery is impaired with consequent mitochondrial dysfunction [138]. Aβ binds to the mitochondrial-matrix protein Aβ-binding alcohol dehydrogenase (ABAD) [139]. Aβ has been shown to inhibit cytochrome oxidase [140,141], and α-ketoglutarate dehydrogenase activities in mitochondria [142]. Overexpression of α-synuclein impairs mitochondrial function and increases oxidative stress [143]. It has been suggested that the prion protein has a neuroprotective function by acting as an antiapoptotic factor that inhibits the mitochondrial apoptotic pathway [144], by preventing the permeability of the mitochondrial membrane.

Research in this field is largely in progress, but the detection of molecular targets of neurodegenerative proteins at level of the mitochondrial matrix and delineation of the role of metal ions in modulating such interactions may open a wide range of potentially and clinically useful perspectives.

At least two types of proteins contributing to neurodegenerative diseases, the amyloid precursor protein and prions, could unambiguously place a role in copper homeostasis or at least in copper biological activities.

3.5.1. Copper and the amyloid precursor protein (APP)

APP belongs to the family of glycosylated transmembrane proteins, it is quite abundant in the brain. APP constitutes a family of several isoforms having different number of amino acid residues. There are several distinct domains within APP structure, including copper (CuBD) and zinc (ZnBD) binding domains, and the toxic peptide amyloid (Aβ) region (Fig. 11) [145].

According to the animal studies (*APP-knockout* mice) APP seems to play some role in copper and iron homeostasis [146]. One of the possible function of APP is copper transport from the extracellular to intracellular space [147,148].

The toxic fragment of APP which is directly involved in the formation of the Alzheimer plaques is Aβ peptide generated during proteolytic processing of APP by two proteases β- and γ-secretases (Fig. 12) [10,143,140,149].

The APP CuBD is located in the N-terminal cysteine rich domain next to the growth-factor like region (Fig. 11) [150,151].

Expression of APP seems to be a key modulator of neuronal copper homeostasis. APP knockout mice were showing increased copper levels in the brain [152]. The CuBD of APP may also influence Cu(I)-mediated neurotoxicity [153,154]. An increase of copper concentration influences the APP metabolism reducing the Aβ production and increases the level of membrane bound secreted APP [155]. Within cysteine rich region of APP there are three His residues. According to the NMR data at least two of them could be involved in metal ion binding (His147 and His151) [149]. The involvement of all three residues is also likely [156], although the steric effects derived from the protein structure could exclude this possibility. The copper-binding site is close to tetrahedral stabiliz-

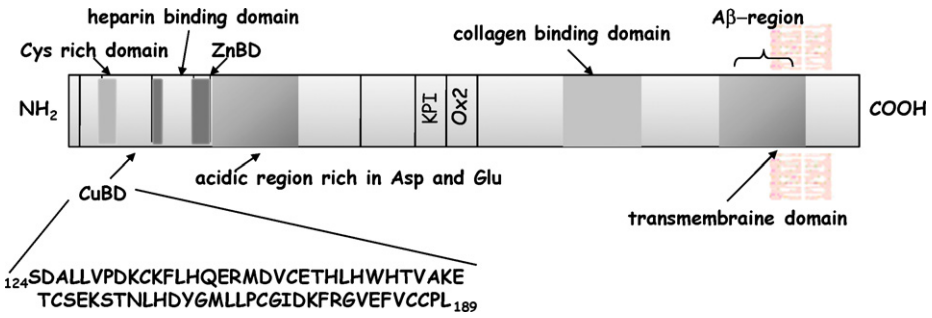

Fig. 11. Schematic representation of APP domains.

ing Cu(I) and allowing neurotoxic redox cycling between Cu(I) and Cu(II) [151]. The interesting feature of the copper binding in APP is its exposure to solvent not similar to normal copper proteins. However, this surface exposure reminds the case of Cu(I) sites in copper chaperon proteins [157]. The general information available for APP may indicate that this protein may behave both as chaperon or transporter (excretion via liver) protein for copper ions [15,149].

Aβ peptide critical for Alzheimer disease is found as a major species within the senile plaques (SP) in the brain. The studies on the intact plaques strongly suggest that Aβ is a metalloprotein species *in vivo* [158]. The human Aβ contains three His residues having very attractive imidazole moieties to coordinate metal ions. Both Zn(II) and Cu(II) are bound to Aβ via three His residues and N-terminal amino nitrogen forming very stable species [159,160] (see Ref. [12] for general discussion). The presence of three His could be critical for peptide aggregation process as in the case of rat Aβ having only two His residues in the peptide sequence aggregation is strongly diminished [161–163]. Copper and iron interactions with Aβ mediate the toxicity of the peptide in the cell culture. These systems catalyze generation of H_2O_2 via reduction of metal ions using O_2. Peroxide in the presence of Cu(I) and Fe(II) generates hydroxyl radical and then products of the oxidative stress. Such oxidative damage is typical for Alzheimer neuropathology [164].

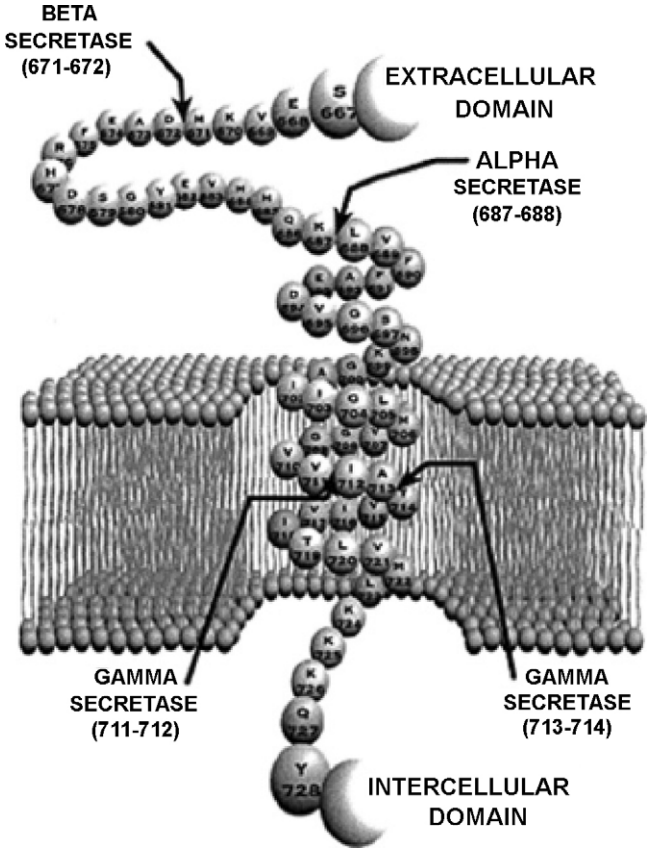

Fig. 12. Schematic view of the membrane spanning and cleavage sites of APP. (Reprinted with permission from Ref. [145].)

3.5.2. Copper and the prion protein

"Mad cow disease" cases in UK, described by all possible media, made the prion proteins one of the most famous molecules in the middle of the 1990s of the last century. Prion diseases have been known in fact for around 300 years (scrapie among sheep was described first at the beginning of the XVIII century). Very few cases are found every year among humans, however, the possibility of inter-species transmission (e.g. from cattle to human) resulting in infection and in the case of human population ending with fatal variant Creutzfeldt–Jakob disease [165], provided huge publicity to this unusual molecule among researchers.

Prion protein (PrP) is a normal cellular moiety expressed by many different cells (PrP^C), found in high amounts in neurons. PrP^C is a glyco-protein anchored by GPI (glycosyl-phosphatidylinositol) to the cell membrane (within the rich in cholesterol raft [166]). PrP^C is a protease sensitive, α-helix containing protein of unknown yet biological function. It can change its conformation to the pathological scrapie form PrP^{Sc} resistant to proteinase K and being quite rich in the β-sheet structure [167]. While the biological functioning of PrP^C is not yet clear PrP^{Sc} aggregates and seems to play a critical role in the development of pathology. To develop disease the permanent supply of PrP^C is needed and PrP^{Sc} is most likely used as a matrix-inducing change to the conformation of the regular protein via the template-directed refolding mechanism [168].

The structural studies of prion proteins both NMR [169–172] and X-ray crystallography [173] clearly show very specific domains, the three α-helical regions, two short β-pleated segment and a long flexible unstructured N-terminal tail up to residue 120 (Fig. 13) [174].

The metal which seems to be most strongly connected to the biology of prions is copper [175,176]. It seems to be clear that copper binds PrP^C within the octarepeat region [177–179] and the imidazoles of four His residues are the anchoring binding sites [180]. The binding of Cu(II) to PrP attached to the cell membrane results in the clathrin-dependent endocytosis [181]. Internalization of the PrP induced by copper may indicate possible biological involvement of PrP in the copper transport from extracellular to intracellular space (Fig. 14) [182].

The other feature of the Cu–PrP system which could be biologically relevant is its antioxidative activity [183]. Cu(II) bound to PrP acts as superoxide dismutase (SOD) protecting against radical oxygen species (ROS). The SOD activity depends strongly on the binding mode of Cu(II) to protein or its fragments (Fig. 15) [184]. Thus, two basic biological functions of prion proteins could be copper transport and SOD activity.

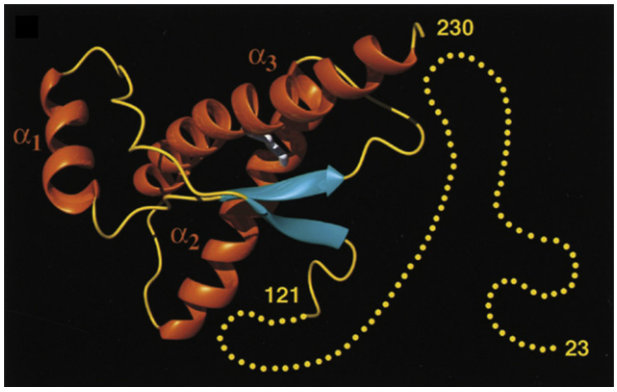

Fig. 13. Cartoon of the three-dimensional structure of the intact human PrP (23–230). (Reprinted with permission from Ref. [174].)

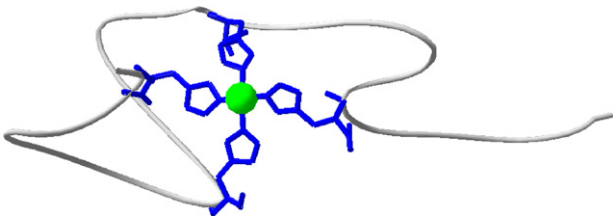

Fig. 15. Schematic representation of structure of Cu^{2+} complex of octarepeat tetramer.

4. Iron

Iron is a functional component for the machinery that transports dioxygen and for many electron transfer enzymes. Though abundant in the environment, its very low solubility in water at physiological pH limits its direct use by living organisms. Moreover the toxicity associated with free iron poses a dramatic challenge for most organisms requiring adequate amounts of iron for critical biological processes. It follows that an imbalance of the body iron determines pathological conditions [185,186]:

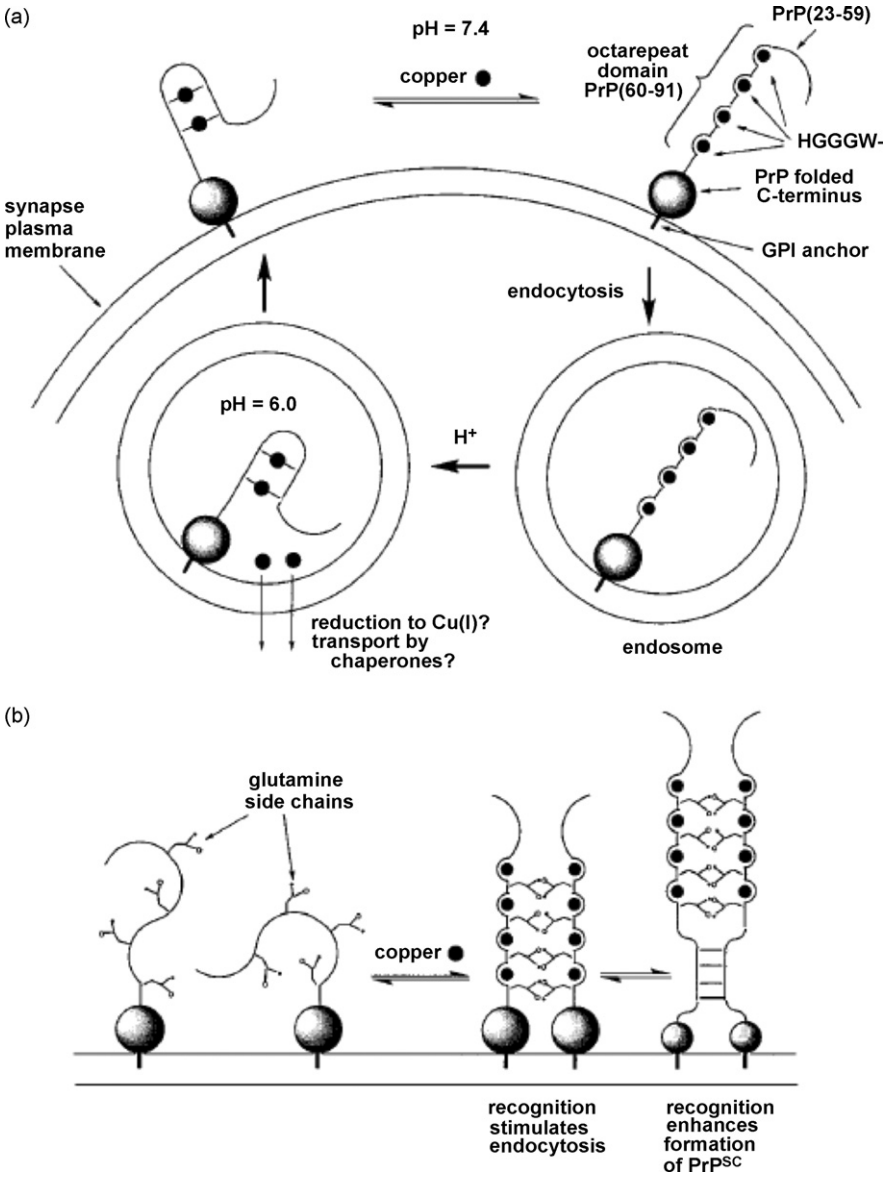

Fig. 14. Scheme of hypothesis: (a) copper transport to intracellular space; (b) internalization of the PrP promoted by copper ions. (Reprinted with permission from Ref. [182].)

- iron deficiency disorders—the most common is anemia, which affects over 30% of the world's population. Inadequate iron intake, blood loss, increased iron requirements, and reduced iron absorption all contribute to the progression of iron deficiency.
- iron overload disorders first discovered with the hemochromatosis associated with excess iron deposition in the liver.

The comprehension of mechanisms underlying transport and homeostatic control of iron in mammals has enormously advanced over the last years. Since the discovery of the hemochromatosis gene (HFE) [187], the comprehension of iron metabolism has progressed through the identification of many new proteins involved in iron transport and regulation, such as the divalent metal transporter 1 (DMT1) [188], hephestin [189], and many others [190].

4.1. Iron homeostasis

Mammals obtain iron from the diet, being heme-iron much more efficiently absorbed than non-heme iron. Absorption is accomplished by enterocytes in the proximal small intestine, near the gastro-duodenal junction. Access to the circulation is modulated by transport through both the apical and basolateral membranes, which is operated by specific transporter proteins and accessory enzymes [191].

Non-heme iron is first reduced to Fe(II), which is dealt by the divalent metal transporter 1 (DMT1), a member of the Nramp family of 12-transmembrane-segment proteins (Fig. 16) [188,192] DMT1 is a proton symporter and requires low pH for efficient transport of divalent metal ions.

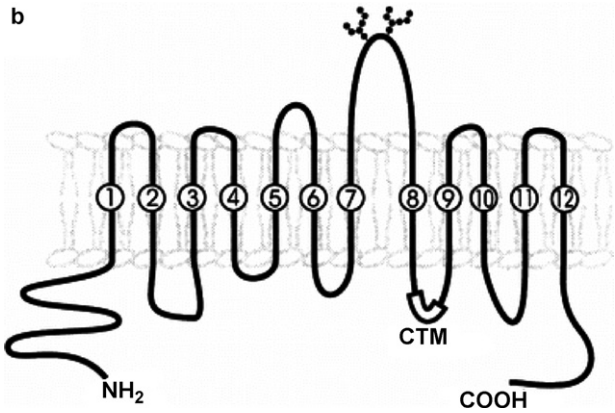

Fig. 16. Twelve-transmembrane-domain model of the DCT1 protein. The 'consensus transport motif is indicated (CTM) in the fourth intracellular loop, and putative *N*-linked glycosylation sites are identified in the fourth extracellular loop. (Reprinted with permission from Ref. [198].)

Heme-iron is directly dealt by the heme carrier protein 1 [193], probably disassembled by heme oxygenase, and iron enters the same storage and transport pathways taken by inorganic iron. Some of absorbed iron is stored in enterocytes' ferritin, and some is exported to the circulation by ferroportin (FPN; also known

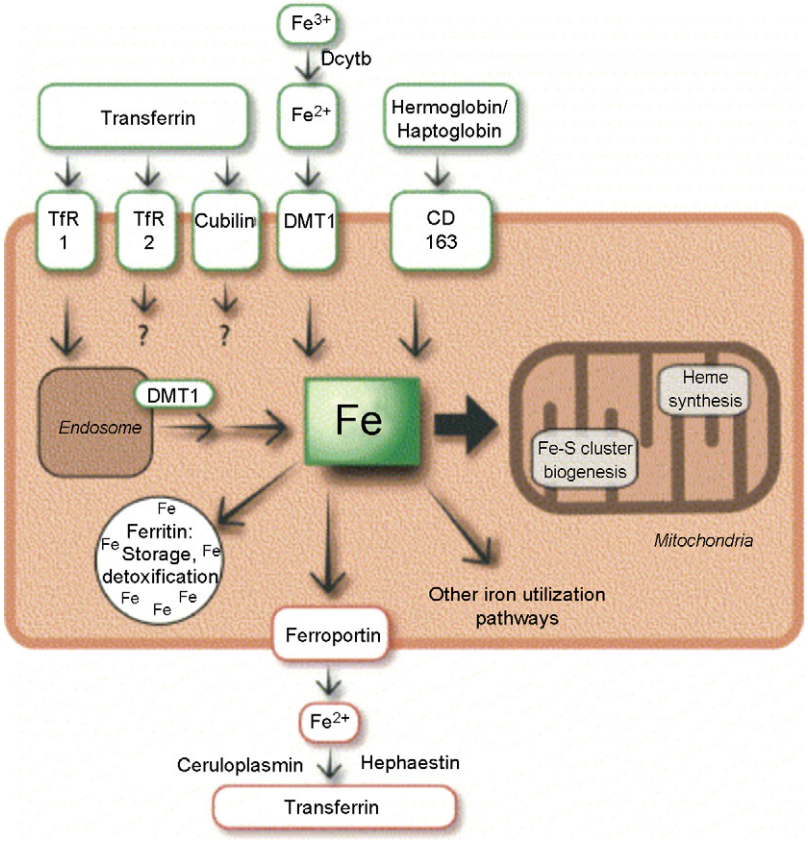

Fig. 17. A generic mammalian cell is depicted with an indication of iron import (top) as well as iron export (bottom) pathways. The transferrin receptor-1 (TfR-1) is ubiquitously expressed, while transferrin receptor-2 (TfR-2) is restricted to hepatocytes, duodenal crypt cells, and erythroid cells. Polarized epithelial cells of the kidney utilize cubilin for transferrin-mediated iron uptake. DMT-1 is implicated in intestinal iron absorption after Fe^{3+} from the diet is reduced to Fe^{2+} by the cytochrome *b*-like ferrireductase (Dcytb). DMT-1 further functions in iron export from the endosome following uptake via the Tf cycle. The hemoglobin scavenger receptor (CD163) plays a role in haptoglobin-mediated hemoglobin uptake of monocytes and macrophages. The only putative iron exporter identified to date is ferroportin, which has been implicated in iron export from duodenal enterocytes, macrophages, hepatocytes, placenta syncytiotrophoblasts, and cells of the central nervous system (CNS). Ceruloplasmin, and its intestinal homologue hephaestin, oxidize Fe^{2+} after cellular iron export for loading onto transferrin. Much intracellular iron traffic is directed toward mitochondria, where the iron-dependent step of heme synthesis and critical steps for Fe–S cluster biogenesis are localized. (Reprinted with permission from Ref. [198].)

as SLC40A1, IREG1, and MTP1). FPN has 10–12 predicted transmembrane segments but bears no homology to DMT1 or other mammalian proteins [194,195].

Iron export requires an associated ferroxidase activity, apparently supplied, in the intestine, by ceruloplasmin and hephaestin [181,196,197].

Absorbed iron is rapidly delivered to transferrin, that is, under normal circumstances, accounts for nearly all serum iron. In normal human subjects, plasma transferrin is only approximately 30% saturated. The different cellular iron uptake and export mechanisms have been recently reviewed [198,199] as schematized in Fig. 17.

The absence of a regulated mechanism for iron excretion determines the necessity of a tight balance between the sites of iron absorption, uptake, transport, storage, and utilization for maintenance of iron homeostasis. This is achieved in several ways. Expression of molecules involved in iron metabolism is regulated both transcriptionally and post-transcriptionally by feedback regulatory events responding to cellular iron status and other stimuli, such as hypoxia, nitric oxide, and oxidative stress.

Many proteins are post-transcriptionally regulated by cellular iron levels, which involves the cytoplasmic iron regulatory proteins (IRP) binding to specific mRNA stem loop structures known as iron responsive elements (IRE) [200]. Under conditions of iron depletion, IRP binds to IRE at the 3′-untranslated region (UTR) of target gene mRNA, increasing its stability and causing an increase in both transcript and protein levels. In contrast, IRP binding to the IRE at the 5′-UTR of target gene mRNA inhibits translation and decreases protein synthesis.

Of the two homologous cytoplasmic IRPs, IRP1 is identical to aconitase, contains a [4Fe–4S] cluster and has aconitase activity [201]. IRP2 shares over 60% identity with IRP1 but it only works as an RNA-binding protein [202].

4.2. Iron in brain aging and neurodegenerative disorders

As in other organs, the brain needs iron for metabolic processes and iron deficiency or excess result into pathological states. Some physiological roles of iron within the brain are in embryonic neuronal development, in myelin formation, in synthesis and metabolism of neurotransmitters, in oxidative phosphorylation and ATP synthesis.

Second only to the liver, the brain contains the highest quantity of iron, *ca.* 60 mg of non-heme iron distributed uniquely according to brain structures [203]. The *substantia nigra* and *globus pallidus* can contain iron levels which exceed hepatic levels [204], containing 3.3–3.8 mM iron in normal adult human brains. These high brain iron concentrations can be attributed primarily to the rapid rate of oxidative metabolism necessary to maintain ionic membrane gradients, axonal transport, and synaptic transmission.

The transport of iron across the blood–brain barrier (BBB) must be regulated, but the permeation mechanism has not been completely clarified so far. The uptake of transferrin-bound iron by TFR-mediated endocytosis from the blood into cerebral endothelial cells is no different in nature from the uptake into other cell types, as schematized in Fig. 18 [205]. After permeation across the BBB or blood–CSF barrier, iron is likely to bind quickly to the transferrin secreted by the oligodendrocytes and choroids plexus epithelial cells, which, diversely from what happens in other tissues, becomes fully saturated with iron. The excess iron will bind to other transporters, including small molecules like citrate or ascorbate. The widespread distribution of TFR in neurons clearly indicates that neurons can acquire iron by means of TFR-mediated uptake of TF-Fe [206].

The discovery of mutations in the genes encoding ferritin light polypeptide (FTL) in ‘neuroferritinopathy’ (or ferritinopathy) and ceruloplasmin in aceruloplasminemia provides convincing evidence that “errors in iron metabolism do indeed have a key role” in neurodegenerative diseases [207,208]. Studies on a mouse line lacking IRP2 and recent observations on the increased onset of AD and PD induced by one or more HFE gene mutation also strongly support the conclusion that iron dyshomeostasis induced by genetic factors (gene mutation or absence) is one of the initial causes in neurodegenerative diseases (Fig. 19) [209].

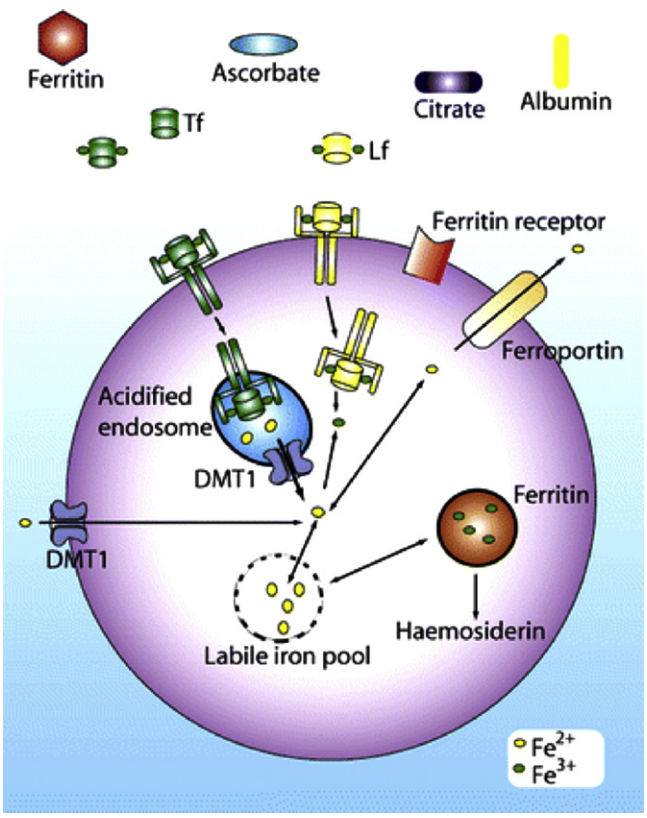

Fig. 18. Iron homeostatic mechanisms in neuronal cells. Cerebrospinal fluid and brain interstitial fluid contain numerous iron-binding molecules represented here in the extracellular space. Iron can enter cells in its trivalent form when bound to transferrin (Tf), lactoferrin (Lf), or ferritin via the respective receptors for these molecules. Divalent metal transporter-1 (DMT1) found on the cell membrane allows iron to enter in its divalent form. Transferrin-bound iron entry is described in detail in the text. Once iron is released into the cytosol it can enter the labile iron pool where it is available for biological processes and is chelatable. Iron is stored within ferritin and can later be incorporated into hemosiderin or be released to enter the labile iron pool. Within the cytosol, ferritin complexes, and endosomes (ferroxidase proteins) oxidize iron for storage or export. Iron can be released from cells via ferroportin or even bound to transferrin, although little is known about iron export from neuronal cells. (Reprinted with permission from Ref. [205].)

Recent observations that HFE mutation is closely associated with onset of AD and PD further support this viewpoint. HFE protein is a membrane protein that can influence cellular iron uptake [210]. HFE mutations represent a risk factor or genetic modifier for AD or PD [211]. HFE protein has recently been localized to the reactive astrocytes and neurons in the brains of patients with AD [212]. It follows that iron imbalance in the brain determined by HFE mutation contributes to AD, and also that disrupted iron metabolism may be a primary risk factor for neurodegeneration and oxidative stress in AD. Case reports of PD and parkinsonism in association with hemochromatosis have also been published [213].

Brain iron unpaired regulation may result also from the disrupted expression of brain iron metabolism proteins induced by non-genetic factors. These currently undetermined factors might disrupt normal control mechanisms of protein expression [214], and lead to iron imbalance in the brain, and then induce oxidative stress and neuronal death in some neurodegenerative disorders (Fig. 20).

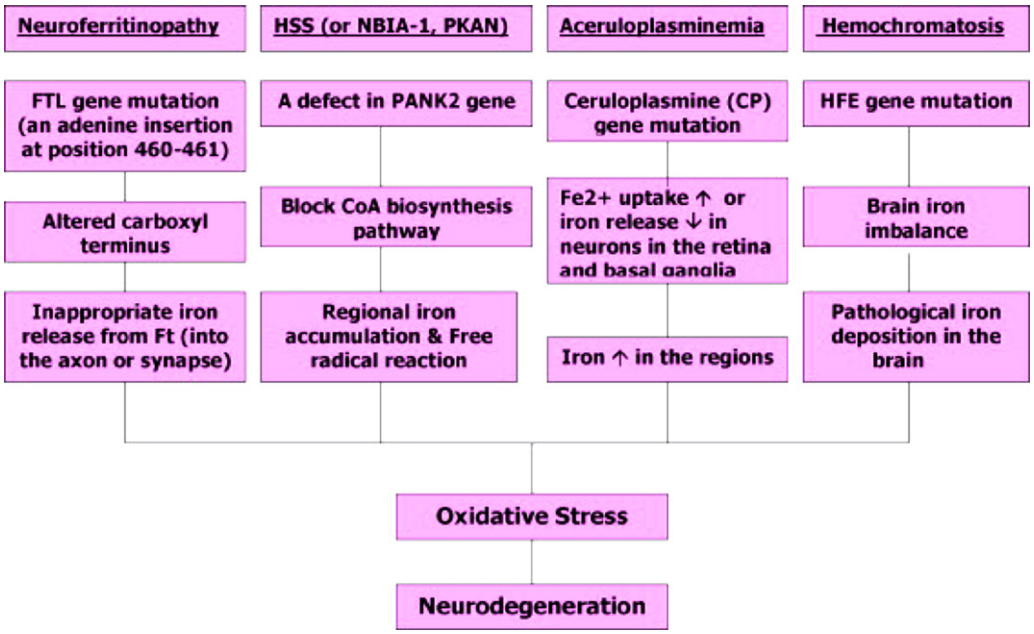

Fig. 19. Role of genetic factors-induced brain iron misregulation in the development of neurodegenerative disorders. *Abbreviations*: FTL, ferritin light polypeptide; HFE, hemochromatosis protein (the protein mutated in hereditary hemochromatosis); HSS, Hallervorden–Spatz syndrome; NBIA-1, neurodegeneration with brain-iron accumulation-1; PKAN, pantothenate kinase associated neurodegeneration; PANK2, a novel pantothenate kinase; Ft, ferritin; CoA, coenzyme A. (Reprinted with permission from Ref. [209].)

Iron accumulation in the brain occurs gradually over time with concurrent increases in ferritin [203]. Brain iron accumulation may induce neuronal damage even after it has become bound to ferritin because iron can be released in its ferrous form under the acidic conditions present in extracellular fluid and through interaction with components such as excess superoxide radicals and ascorbate. Iron overload results in a large increase in the chelatable free iron pool which is too large to be sequestered by ferritin within cells.

Iron toxicity, largely based on Fenton chemistry, mainly affects the mitochondrial inner membrane respiratory complexes. Fig. 21 shows the pathways involved in iron accumulation and iron-associated toxicity that occur within neuronal cells [197].

Disruption of iron regulatory mechanisms and iron accumulation may contribute to development and progression of neurodegenerative disorders such as AD and PD. Interestingly, normal brain iron content and excessive accumulation both seem to be completely uncoupled from systemic iron levels. Therefore, iron overload conditions that manifest in blood, such as found in hemochromatosis (an inherited disorder of abnormal iron absorption and accumulation) often do not correlate directly with iron accumulation within the CNS, and vice versa.

The brain of Alzheimer's diseased humans is characterized by the accumulation of iron within senile plaques (*ca.* 1 mM) and neurofibrillary tangles, and also by lowered expression of transferrin receptor. As a consequence, these brains are subject to high levels

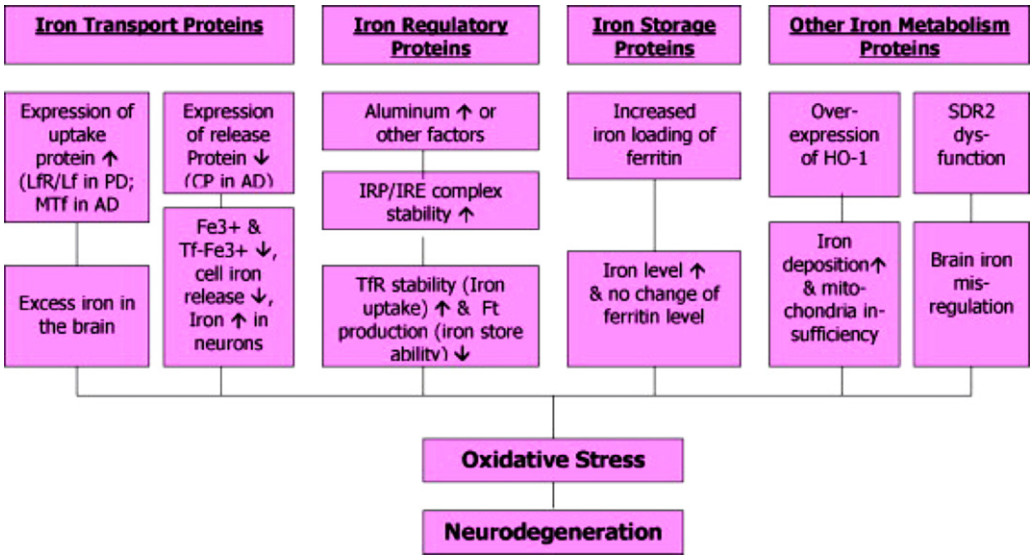

Fig. 20. Scheme for the possible role of non-genetic factors-induced mis-expression of iron metabolism proteins in the development of some neurodegenerative disorders. *Abbreviations*: LfR, lactoferrin receptor; Lf, lactoferrin; PD, Parkinson's disease; p97, melanotransferrin; AD, Alzheimer's disease; NDs, neurodegenerative disorders; DMT1 (DCT1 or Nramp2), divalent metal transporter1; CP, ceruloplasmin; Al, aluminum; IRP, iron regulatory protein; IRE, iron regulatory element; TfR, transferrin receptor; Ft, ferritin; HO-1, heme oxygenase-1; SDR2, stromal cell-derived receptor 2. (Reprinted with permission from Ref. [209].)

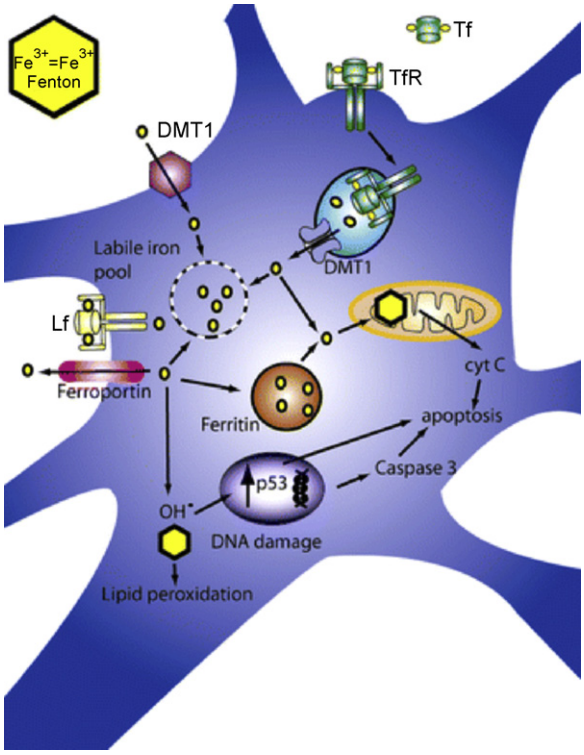

Fig. 21. Representation of iron-induced cell death in neuronal cells. Iron overload or iron accumulation leads to an increase in circulating and intracellular free iron due to saturation of iron-binding, transport, and storage proteins. Circulating free iron can enter cells via the divalent metal transporter-1 (DMT1). Presumably there also exist other mechanisms of non-transferrin bound iron entry. Excess intracellular iron leads to increased production of free radicals which may react with membranes, proteins, and DNA due to saturation of antioxidant molecules. Oxidative damage to DNA stimulates increased expression of p53 and caspase 3, while mitochondrial damage stimulates the release of cytochrome *c*. These events all participate in apoptotic pathways. (Reprinted with permission from Ref. [205].)

of oxidative stress [215]. Iron may also promote Aβ deposition [216], and may affect the enzymatic processing of the amyloid precursor protein [217].

As for PD, dopaminergic cell loss and disease progression are accompanied by the accumulation of high iron concentrations, that are particularly associated with aggregation of α-synuclein (especially the mutated form found in familial PD) within Lewy bodies [218]. An increased iron content can be detected in the substantia nigra of most PD patients and up to a 255% increase in intracellular iron concentration has been observed in post-mortem PD brains [219]. Together with iron accumulation, the lowered expression of ferritin within the *substantia nigra* of PD patients results into oxidative stress and decreased glutathione levels, thus directly contributing to dopaminergic neuronal toxicity.

Many other neurodegenerative diseases, such as Huntington's disease, Friedreich's ataxia, amyotrophic lateral sclerosis, and prion disease, correlate with changes in iron metabolism yielding iron accumulation [220]. All these disorders share cell-specific apoptotic death [221], and the presence of abnormal concentrations of iron in the affected areas [211]. It is still a matter of scientific debate whether iron accumulation is causative to or resulting from the individual disorders. Most likely, iron-induced oxidative damage acting on mitochondria contributes to the cellular death mechanism that results from an attenuation of respiratory chain activity. The significant reduction in transferrin levels, observed in PD and AD, may act as a contributory factor to increase iron concentrations [222].

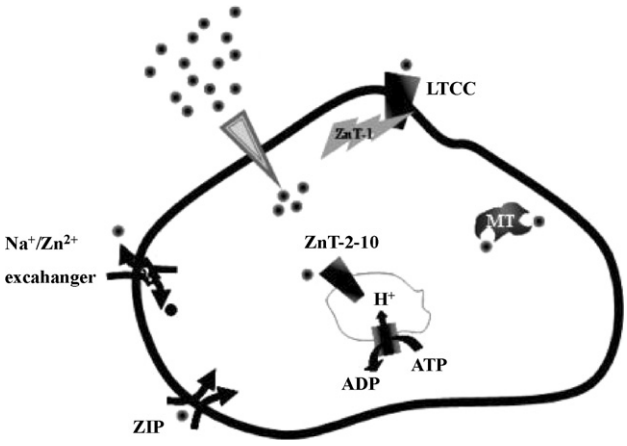

Fig. 22. Scheme of the mechanisms of zinc homeostasis in mammalian cells. Zinc is distributed at large transmembrane and vesicular gradients. These are generated by the orchestrated activity of multiple zinc transporters and regulators of zinc transport. For the sake of simplicity, the ZnT2-10 have been illustrated on a single compartment although they may be found on multiple organelles. (Reprinted with permission from Ref. [223].)

5. Zinc

5.1. Zinc homeostasis

After iron, zinc is the second most abundant trace element in the body. The highest concentrations of zinc are found in the brain and, also, in pancreatic β cells. In the brain, chelatable, i.e. removable by chelating agents, zinc highly occurs in the hippocampus, amygdala, and in the cortex. Beyond this, zinc is tightly bound by many intracellular enzymes. Zinc, in its ionic form, can also exert important modulatory effects on neurotransmission and synaptic function, as well as regulate many signalling pathways [223,224].

3–10% of all mammalian proteins bind zinc as an essential cofactor for folding, conformational change, or activity. Zinc deficiency is therefore accompanied by consequences as dramatic as erosion of the gastrointestinal tract, or skin lesions, or cardiac failure, or malformations of brain and the male reproductive system [225]. However, zinc, especially its "free" or loosely bound form, is nevertheless very toxic to mammalian cells [226]. For example, evidence has been reached that zinc is a key factor in neuronal death associated with ischemia and seizures, as well as in other neurological disease states [227], such as in the formation of Alzheimer's β-amyloid plaques [228], or in the degeneration of pancreatic β-cells [229].

The dual aspects of cellular zinc are dealt by distributing the metal in highly regulated gradients across the plasma membrane and among intracellular compartments [223]. In the same way, an adequate supply of zinc must be available for insertion into proteins, while preventing its accumulation and the potentially devastating effects it can readily initiate.

The zinc gradient present at the plasma membrane is even higher than that of calcium [230], as exemplified by:

- zinc concentrations in the synaptic cleft (μM range) as compared with the pM intracellular free-Zn(II) concentration;
- mM concentrations of Zn(II) existing in the intracellular synaptic and secretory vesicles.

The complexity of zinc homeostasis is reflected by the large number of proteins dedicated to transport and buffering of zinc (Fig. 22) [223]:

- at least 10 members of the ZnT (Zn(II) Transporter) family; [231]

- 15 members of the ZIP (i.e. Zn(II)-regulated metal transporter, iron-regulated metal transporter-like protein) family; [232]
- 3 distinct isoforms of metallothionein [93].

Zn(II) is co-released with glutamate in many excitatory synapses; this Zn(II) can eventually enter neurons through NMDA and AMPA/kainate receptors, or through voltage sensitive calcium channels and Zn(II) transporters [233,224]. The neuronal concentration of free Zn(II) is maintained at low levels, probably in the fM range, with the aid of extrusion, buffering, and sequestration systems [224].

Two main mechanisms account for the build-up of Zn(II) gradients [223,234]:

1. a primary ATPase pump;
2. a secondary active mechanism using sodium gradients.

Though demonstrated in bacteria [235], and in Arabidopsis [236], there is still no evidence for a Zn(II) pump in either yeast or mammalian cells. As a consequence, the secondary active mechanism has been suggested for the formation of Zn(II) gradients in neurons, where a putative Na(I)/Zn(II) exchanger has been suggested to promote Zn(II) efflux against a 500-fold transmembrane gradient [237].

Zn(II) transporter proteins (ZnTs) are members of the CDF (cation diffusion facilitator) family made of 9 human CDF genes (also known as SLC30 genes): 10 ZnTs (ZnT-1–10) have been identified so far [238]. ZnT-1 and ZnT-3 are highly expressed in the brain. ZnT-1 is present on the neuronal plasma membrane and is highly expressed in brain regions rich in synaptic Zn(II). Expression of ZnT-1 is correlated with the appearance of synaptic Zn(II) and it has been shown to reduce Zn(II) toxicity. ZnT-3 also occurs in brain regions rich in chelatable Zn(II) and a possible role has been suggested for uploading Zn(II) into synaptic vesicles [235,224,239,240].

The over 300 members SLC (SoLute carrier) group of membrane transport proteins are organized into 47 families [241]. Transported solutes are very diverse from each other (both charged and uncharged organic molecules, inorganic ions). SLCs are integral membrane proteins containing hydrophobic transmembrane alpha helices connected by hydrophilic intra- or extracellular loops. SLCs may be functional as monomers as well as homo- or hetero-oligomers. Members within the individual SLC family have greater than 20% sequence homology. The SLC group include examples of transport proteins that are:

- facilitative transporters (solutes flow downhill with the electrochemical gradients),
- secondary active transporters (solutes' flow uphill against the electrochemical gradient is coupled to transport of a second solute flowing downhill with its gradient).

Most members of the SLC group are located in the outer cell membrane, but some members are located in mitochondria (most notably SLC family 25) or other intracellular organelles. The SLC group 39 contains 14 orthologs that are zinc transporters also called ZIP proteins.

Metallothioneins (MTs) exert a critical role in buffering cytosolic Zn(II). MT-3 seems to be particularly relevant to neuronal Zn(II) homeostasis in critical brain regions such as the hippocampus where it is abundantly present in the same hippocampal glutamatergic terminals that are also strongly enriched in vesicular Zn(II). As already said, the MTs are small proteins (61–68 amino acids) with a highly conserved sequence of 20 Cys residues yielding two domains for Zn(II) binding. The Zn(II)/cys cluster regions can be readily modulated by changes in pH or, better, by changes in the redox state. For example, oxidative stress, while interfering with zinc binding to MTs, acts as a key regulator of $[Zn(II)]_i$ homeostasis. As a matter of fact, cellular oxidants may promote Zn(II) release from MTs and reducing agents may facilitate Zn(II) binding [224,242].

The sequestration of cytosolic Zn(II) is mainly dealt by mitochondria that are also very important for clearing of cytosolic Ca(II) overloads. Uptake of Zn(II) by mitochondria is apparently and largely mediated by the Ca(II) uniporter. Mitochondria are thought to have a high-Zn(II) uptake capacity such that (i) blocking of this process determines a significant elevation of cytosolic $[Zn(II)]_i$ and (ii) the mitochondrion itself may provide an intracellular store for $[Zn(II)]_i$ mobilization. Under resting conditions, the Zn(II) sequestered by mitochondria may be re-released into the cytoplasm in a Ca(II)-dependent fashion [224,243].

5.2. Zinc in brain aging and neurodegenerative disorders

In the central nervous system, Zn(II) has been implicated in the regulation of many channels and receptors, but the cation can also act as a trigger for neuronal loss in several neurological conditions [216]. Deregulation of neuronal Zn(II) homeostasis is believed to be strictly connected to mitochondrial dysfunction and oxidative stress, making the cation a possible contributor to the activation of pathophysiological pathways involved in brain aging and/or neurodegeneration. In fact, exposure to high-Zn(II) concentrations both *in vitro* and *in vivo* promote neuronal death, and elevated neuronal $[Zn(II)]_i$ levels are linked to the neuronal loss observed in excitotoxic conditions such as epilepsy, ischemia, and traumatic brain injury [216].

The hypothesis has been made that Zn(II)-related neuronal loss can be attributed to the injurious trans-synaptic movement of the cation from Zn(II) releasing terminals to post-synaptic neurons, a process that has been called "Zn(II) translocation" [224]. However, excitotoxic insults are nevertheless able to promote intra-neuronal accumulation of injurious Zn(II) [244], indicating that neurons possess intracellular sites from which Zn(II) can be mobilized and promote injury.

Zn(II) can trigger ROS production through mitochondrial and extra-mitochondrial pathways. Studies in isolated mitochondria indicate that zinc [224]:

- inhibits cellular respiration by interfering with the activity of the electron transport chain (ETC);
- inhibits complex III at cytochrome bc_1;
- inhibits complex I by inhibiting α-ketoglutarate dehydrogenase (KGDHG) [245].

ROS can also be generated by extra-mitochondrial pathways that include an increased activity of NADPH oxidase (widely expressed in central neurons) and the induction of neuronal nitric oxide synthase (nNOS) which, together with superoxide, produces peroxynitrite ($ONOO^-$). In a feed-forward cyclic manner, Zn(II) trigger ROS generation and cellular oxidation promotes further $[Zn(II)]_i$ release.

As already quoted, the metallothioneins apparently constitute one of the major targets of ROS-dependent Zn(II) release, in spite of the intrinsic antioxidant properties of these proteins. In principle, the ability to release Zn(II) upon changes in the cellular redox state renders MTs a reservoir of readily available Zn(II) under conditions of oxidative stress. The latest observations strongly suggest that nitrosative stress can critically modulate $[Zn(II)]_i$ mobilization. Nitric oxide or peroxynitrite interact preferentially with MT-3 and promote Zn(II) release from MTs both *in vitro* and *in vivo* [246].

Zn(II) has multi-directional effects on cellular physiology and can activate multiple death pathways within neurons, since Zn(II) modulates both necrosis and apoptosis. Once thought to be mutu-

ally exclusive, the two death pathways are nowadays agreed to be actually co-existing, with the affected status of cellular energy levels determining which process prevails in a given cell. Zn(II) can potently disrupt mitochondrial functions by triggering the release of ROS, modulating the opening of the mPTP, and promoting the release of pro-apoptotic factors.

Zn(II) has also been linked to impairment of neuronal metabolism through inhibition of key enzymes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphofructokinase, and NAD^+ glycohydrolase. Inhibition of GAPDH, efficiently determined by submicromolar cytosolic $[Zn(II)]_i$, leads to ATP depletion, and neuronal death. ROS-induced $[Zn(II)]_i$ accumulation yields activation of specific potassium channels, a key event in neuronal apoptosis [247].

Extra-mitochondrial and mitochondrial pathways can possibly converge: for instance, the activation of cytosolic poly (ADP-ribose) polymerase (PARP) modulates the release of the apoptosis-inducing factor (AIF) from mitochondria. In turn, cytosolic AIF promotes the release of Cyt-*c*, and initiates the apoptotic cascade [248].

The mobilization of intracellular Zn(II) under conditions of oxidative stress may provide the link between necrotic and apoptotic pathways. In fact, it has been observed in intact neurons that $[Zn(II)]_i$ mobilization by cellular oxidants affects the mitochondrial membrane potential; whereas, in isolated mitochondria, comparable [Zn(II)] rises trigger the mPTP opening [229]. Conversely, Zn(II)-induced mitochondrial ROS generation is expected to promote further Zn(II) release. It follows that the two pathways can work together in a vicious cycle of neuronal injury.

Aging is universally accompanied by oxidative stress, alterations in cell metabolism, accumulation of proteinaceous deposits, nucleic acid damage, etc. Brain aging is specifically associated with progressive neuronal loss, cognitive impairment, and enhanced susceptibility to neurological diseases.

How can an impaired Zn(II) homeostasis affect brain aging? Given the potent role played by Zn(II) in oxidative stress and mitochondrial dysfunction, the following feed-forward process has been suggested:

- oxidative stress induces Zn(II) release from MTs;
- Zn(II) uptake leads to mitochondrial dysfunction and increased mitochondrial ROS generation;
- mitochondrial ROS further promote Zn(II) mobilization from MTs and cytosolic $[Zn(II)]_i$ rises;
- $[Zn(II)]_i$ activates mitochondrial Zn(II) sequestration and apoptosis;
- under chronic conditions, this cycle is further amplified and perpetuated by increased mtDNA mutations (also linked to Zn(II)-induced oxidative stress) and expression of defective proteins, making mitochondria less efficient in ROS recycling.

This being the case, a central role is emerging for metallothioneins that act as key determinants of $[Zn(II)]_i$ homeostasis. In fact:

- MTs are highly expressed in astrocytes and hippocampal neurons in the aging brain [249];
- MTs are strong antioxidants and protective factors;
- increased MT expression may represent an endogenous protective response against prolonged exposures to inflammatory agents.

Supporting this idea, increased serum levels of pro-inflammatory cytokines like IL-1, IL-6 and TNF-α have been linked to aging [250], leading to define "inflam-aging" the persistent age-related state of inflammation.

6. Conclusions

Metal ions, such a copper, iron and zinc, are essential for all living organisms and participate in a wide variety of metabolic processes in the cells. However, their concentrations in the body tissues must be strictly and tightly regulated because of their redox (Cu and Fe) activity contribute to the production of toxic free radicals that can react with various organic substrates such as lipids, proteins, DNA, etc. Oxidation of these biomolecules can damage them, disturbing normal functions and may contribute to a variety of disease states. Cells posses perfect control mechanisms to maintain metal concentrations on correct level by coordinately regulating their absorption process, recycling and storing up. The coordinate control of ions uptake and storage is tightly regulated by the feedback system involving regulatory proteins and metallochaperons. The major consequences of metal dyshomeostasis are mitochondrial dysfunction, oxidative stress and mitochondrial genomic damage which enhance activation of the apoptotic machinery. A lot of evidence indicates also that transition metal-mediated abnormalities play a crucial role in many neurodegenerative disease pathogenesis. This review is a general overview of cellular copper, iron and zinc metabolism and regulation with describing the function of key proteins involved in their homeostasis and alterations in Alzheimer, Parkinson and prion diseases. Many fundamental questions remain about activities and regulations of metal ion proteins such transporters and regards the existence of functional cross between the metal transporters and other ion transport mechanisms. The molecular understanding basis of the metal homeostasis and regulations in the cells are critical in identifying the underlying causes for diseases pathophysiology, providing proper diagnosis and treatments. It is also necessary for the development of new therapeutic agents able to treat and prevent their occurrence.

Acknowledgement

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