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Review

Metal ions as modulators of protein conformation and misfolding in neurodegeneration

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ABSTRACT

Protein misfolding and conformational changes are a cornerstone of neurodegenerative diseases involving formation and deposition of toxic protein oligomers. Although mutations favor protein aggregation, physiological factors such as labile metal ions within the cellular environment are likely to play a role. Metal ions such as calcium, zinc and copper are key players in brain neurobiology, their homeostasis is altered in most neurodegenerative conditions and they are found within proteinaceous inclusions from patients. In this review we will elucidate the intricate interplay between protein (mis)folding and metal

Abbreviations: Aβ, amyloid-β peptide; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; CJD, Creutzfeldt–Jakob disease; CNS, central nervous system; CSF, cerebrospinal fluid; HD, Huntington's disease; IDP, intrinsically disordered protein; MT, metallothionein; NMDA, N-methyl-D-aspartate; NMDAR, NMDA receptor; PD, Parkinson's disease; PHF, paired helical filaments; PrP^{C}, cellular prion protein; PrP^{res}, prion protein protease resistant conformation; PrP^{SC}, scrapie form of the prion protein; RAGE, receptor for advanced glycation endproducts; ROS, reactive oxygen species; SOD1, superoxide dismutase 1; TSE, transmissible spongiform encephalopathy; ZIP, zinc importing protein; ZnT, zinc transporters.

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ions, discussing how metals modulate protein folding and influence protein energetics, with specific attention on conformational changes and structural fluctuations. In particular, the influence of metal ion dyshomeostasis during neurodegeneration and the effects of the unique physical and chemical properties at the synaptic environment will be discussed in the context of protein deposition. These interactions will be illustrated by specific examples of proteins involved in neurodegenerative diseases including α-synuclein, tau, superoxide dismutase 1, the prion protein and the amyloid-β peptide. With this approach we aim to systematize the effects of metal ions on protein conformers and illustrate pathways through which they modulate protein aggregation, under different conceptual mechanisms that bridge protein structure, metallochemistry and neurobiology.

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1. Metal ions and protein folding

Metal ions are essential for life and metal-binding proteins (*i.e.* metalloproteins) constitute around one third of the proteome. Among others, protein-bound metal cations such as iron (Fe^{3+}/Fe^{2+}), zinc (Zn^{2+}), copper (Cu^{2+}/Cu^{+}), calcium (Ca^{2+}), magnesium (Mg^{2+}) or manganese (Mn^{2+}/Mn^{3+}) are determinant in affording functions related to electron transfer processes, catalysis and stabilization of the protein structure [1]. The association between a metal ion and a protein involves electrostatic and coordinative interactions established by the metal cation with specific combinations of residues that constitute metal coordination motifs within the binding site. Alternatively, this interaction can be established indirectly *via* a metal cluster (*e.g.* an iron-sulfur cluster) or a larger chemical group (*e.g.* a heme cofactor). Whatever the circumstance, metal–polypeptide interactions influence the energetics of protein folding and modulate protein conformation and dynamics [2,3]. Altogether, these effects are at the core of the influence of metal ions over protein deposition processes in neurodegeneration and its fundamentals will be overviewed in this section.

1.1. Energetics of protein folding and metal ion binding

Protein folding is the physical process through which a polypeptide acquires a particular three dimensional conformation. The folding of a protein results from the establishment of a set of interactions which are dictated by the composition of the protein primary sequence [4]. The conformer obtained at the end of this process corresponds to the so-called native state, which is the most stable structure under physiological conditions. The energetics of protein folding can be described by the central thermodynamic equation

$$\Delta G = \Delta H - T\Delta S \quad (1.1)$$

where ΔH represents the free energy change arising from additive contributions resulting from chemical bonding and interactions, whereas $-T\Delta S$ accounts for the free energy changes resulting from variations in the degree of order within the system. Dominant driving forces towards the unfolded state comprise predominantly the high configurational entropy (ΔS_{conf}) of the unfolded protein conformers and van der Waals interactions with the surrounding solvent water molecules. Stabilization of the folded state is generally accepted to result mostly from the 'hydrophobic effect' which refers to the set of non-polar interactions ($\Delta G_{non\text{-}polar}$) that nucleate the folding process [5]. However, other types of interactions also contribute to free energy changes that result in stabilization of the native state, such as: loss of configurational entropy due to the decrease of accessible conformers and protein movements ($-T\Delta S_{conf}$), electrostatic interactions established between polar residues and surrounding water molecules (ΔG_{elect}), and van der Waals interactions which are optimized upon formation of the tight protein core (ΔG_{vdW}) [6,7].

The stability and dynamic properties of the native state are also influenced by other interactions such as those resulting for example from the formation of disulfide bridges or from the association of a cofactor to the protein. Although these interactions are in a way extrinsic, their formation depends on the primary structure and contributes to free energy changes (ΔG_{ext}). Protein–metal ion associations fall into this category: upon metal binding the protein fold undergoes a series of adjustments whose energetics matches that of the metal–ligand interaction [1]. This results from a compromise on coordination numbers, bond lengths and angles which are imposed both on the metal and on the protein fold. The protein–metal interaction is determined by the equilibrium binding constant involving the coordinating groups and by interactions with outer sphere neighbors which contribute to set the particular structural scaffold that allows for a specific metal to be selected over another [1,8]. In this sense, the protein fold itself has the ability to exert a selective power over binding of a particular metal. One mechanism through which this selection can be achieved is by fine-tuning coordinative environments so as to change the internal dielectric constant within the metal binding site, as the energetic cost resulting from the transfer of a metal from an aqueous environment into a low dielectric constant medium such as a binding pocket can be rather large. One consequence of this effect is that different metals can bind to the same coordination groups in the same geometric environment. Overall, the coordination of a metal to a protein depends on the dielectric environment and solvent accessibility, the metal cation properties in respect to accepting ligand charges, and on the chemical characteristics of the ligands [8]. The vast majority of protein–metal interactions involve both electrostatic interactions and coordination bonds, but in around one third of metalloproteins metal binding is essentially coordinative [3,9]. The affinities of individual amino acid chains to certain oxidation states of metals results in a somehow typical selectivity pattern: ligands include histidine, cysteine, aspartate and glutamate residues, but oxygen, nitrogen and sulfur atoms from various residues are also involved [1,8,9]. In proteins, these interactions engage certain combinations of amino acids (from two to seven ligands) that assure proper metal insertion in a catalytic or structural site (Table 1).

The protein fold and metal ion coordination geometry are thus energetically interdependent as a result of the chemical nature of the coordination chemistry, the physical constraints imposed by the protein structure and also of the kinetic stability of the metal protein association. The latter point is of particular relevance since even for a thermodynamically stable interaction, a very low activation energy for dissociation will result in frequent metal exchange reactions, making the site kinetically labile. This is for example the case of calcium binding, whose signaling role relates to its ability to bind/release fast to/from sites of intermediate strength. If multiple metal binding sites are involved, or even if protein conformational changes take place upon metal release, then the energetic balance gets more complex as a result of cooperativity phenomena. In these cases, metal binding and release at one site propagates a conformational effect that affects the coordination sphere and binding

Table 1
Typical coordination environments of selected metal cations in proteins.

Metal cation	Bond stability	Coordination number	Side chain ligands	Coordination geometry
Zn^{2+}	High	3	His, Cys, Glu	Severely distorted tetrahedron
Cu^{+}	High	3,4	His, Cys, Met	Severely distorted tetrahedron
Cu^{2+}	High	3,4	His, Cys	Distorted square planar
Ca^{2+}	Intermediate	7 (8)	Glu, Asp	Pentagonal bipyramid, trigonal prism, distorted octahedron
Fe^{2+}	Low	4–6	His, Glu, Asp Cys	Distorted octahedron tetrahedron
Fe^{3+}	High	4–6	Glu, Asp, Tyr Cys	Distorted octahedron tetrahedron

Adapted from [1,8].

energetics of another site, either by increasing (positive cooperativity) or decreasing (negative cooperativity) its affinity. As detailed in the following section, this energetic crosstalk can be particularly relevant when the metal binds to intrinsically disordered proteins or oligomers.

Overall, the large number of small additive contributions stabilizing either the native or unfolded states that result either from the primary sequence or from protein–metal interactions adds up to an energetic difference averaging only 5–20 kcal/mol in favor of the native state. Consequently, folded proteins are marginally stable [6].

1.2. Protein folding, dynamics and metals ions

Besides influencing protein energetics, metal ion binding also modulates protein dynamics and folding. The process of protein folding can be described using the concept of an energetic landscape, which can be likened to a funnel. According to this view, the funnel edge holds the broad ensemble of unfolded conformers characterized by a high energy content resulting from a large conformational entropy [10,11]. On the other hand, the native conformation lies at the bottom of this funnel, at an energetic minimum achieved as a result of multiple small-magnitude electrostatic and hydrophobic interactions, whose additive effect yields a stabilizing enthalpic component that counteracts the unfolded chain entropy. Rather than resulting from an iteration of all possible combinations through which a polypeptide can be wrapped up, protein folding is in fact a stochastic process which derives from conformational sampling and selection of the most productive conformers that lead to the native conformation [11].

Protein folding pathways can thus be conceptually depicted as trajectories obtained from sliding down this funnel's walls: the inherent conformational fluctuations of the unfolded polypeptide chain allow residues to get in contact with each other. Interactions leading to native like conformations are preferentially selected over those favoring the unfolded conformation; those that afford energy decrease further favor the process thus promoting the cooperativity of the folding reaction. This step involves nucleation–condensation phenomena: upon the interaction of a subset of residues forming a folding nucleus, a narrower set of conformers with native-like architecture is obtained and the native structure is then promptly formed upon coalescence of the rest of the protein around this folding nucleus [12]. The formation of this transition state constitutes an important check point that minimizes misfolded conformations. Such mechanistic scenario is valid for proteins of all sizes, the difference being that whereas small proteins (typically with less than 100 amino acids) fold in a two-state process, larger proteins seem to fold in modules *via* intermediates, as folding takes place independently in different domains of the protein. In these cases the native folded structure is achieved in a final cooperative event during which water is excluded from the protein core and packing of amino acid side chains takes place [13]. Usually protein folding is a relatively fast process and the fact that one specific (correct) fold is achieved in detriment of another (aberrant) is the result of evolutionary pressure which has driven protein sequences to be composed of amino acid strings which maximize favorable interactions [14].

Differences between dynamic properties of protein folds must also be considered as metal ion ligands will affect structure and protein conformations differently. The fact is that not all folds populate either folded or unfolded conformers, as ordered *versus* disordered entities. For some folds there is a clear energetic difference and sharp conformational distinction between the native and unfolded conformers (Fig. 1A). However, the so-called intrinsically disordered proteins (IDP) escape from this paradigmatic view: these proteins (or segments therein) lack either defined secondary or

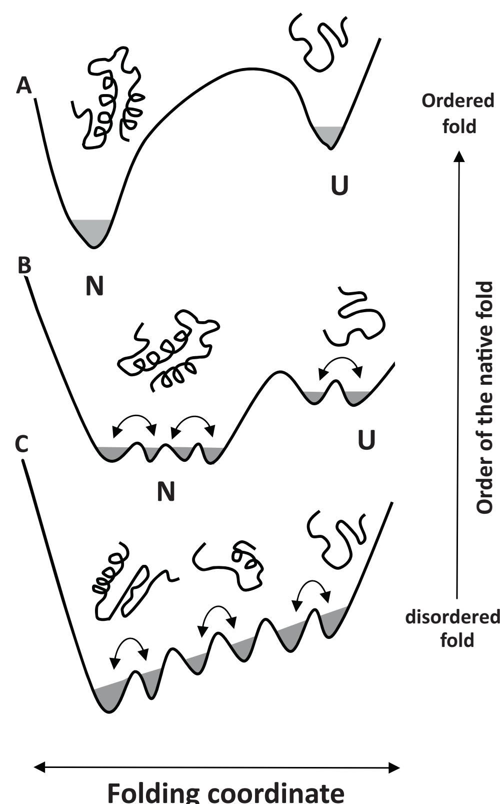

Fig. 1. Energy landscapes of distinct protein folds. Energy landscapes depicting variations of free energy as a function of the folding coordinate are illustrated for: (A) ordered protein folds with defined native (N) and unfolded (U) protein states, clearly separated by an energetic barrier favorable to the N conformer; (B) folds of intermediate order in which local motions yields several conformations around the N and U states, separated by low energy barriers; (C) intrinsically disordered proteins in which there no clear energetic distinction between ordered and disordered conformations.
Adapted from [1].

tertiary structure, existing as structural ensembles [15]. This is to say that, for example, in opposition to a two-state well-ordered globular fold, disordered proteins do not have a single global minimum in the conformational space but rather constantly fluctuate between a multiplicity of structural states which are separated by very low energy barriers (Fig. 1C). Nevertheless, proteins containing disordered regions are involved in vital cellular processes [16,17]. Examples of disordered proteins include many affected in neurodegeneration, such as α-synuclein and the prion protein, as further discussed in the following sections and as recently reviewed in [18]. Other examples include metallothioneins (MT) which rely on metal-thiolate clusters for function, lack defined secondary structure and whose folding into a functional conformation strictly depends on metal ion-coordination [19]. This is a striking example of how metal ion binding to a disordered protein can yield a substantial stabilizing change in the free energy and conformational fine tuning. Many other protein folds have intermediate behaviors, in the sense that local motions within the two extreme states give rise to several conformers associated with the partly ordered and disordered forms which are separated by energy barriers that differ little in energy (Fig. 1B). These are conformational states which are thermodynamically distinct from each other, but structurally similar, and that can be interconverted through thermal fluctuations [20]. Examples of such folds include those of EF-hand containing calcium-binding proteins such as calmodulin or the S100 proteins, which undergo structural fluctuations and have a high conformational plasticity, also as a result of metal binding and release events [21–23]. Whatever the case, both metal ion binding and dissociation to proteins results in a modification of the physical and chemical properties of the protein influencing its dynamic properties, folding and stability.

For metal binding proteins it is clear that the incorporation of the metal ion into the nascent polypeptide modulates both the protein folding landscape and folding trajectories [24,25]. As further discussed in the following sections, metal ion pools in the cell are tightly regulated and a complex protein machinery is involved in the maintenance of metal ion homeostasis; specifically, metal delivery to polypeptides is assured by a specific group of proteins called metallo-chaperones, whose role is to deliver the metal ion to its target holo protein (see *e.g.* [26]). Nevertheless, the mechanisms that mediate metal insertion into newly folded proteins remain essentially unknown for a large number of metalloproteins; however, three generic scenarios can be depicted: (i) co-translational metal ion binding; (ii) post-translational metal ion binding to incompletely folded proteins; and (iii) post-translational metal ion binding to folded apo proteins [9]. These events can be schematically depicted in the perspective of an energetic folding funnel (Fig. 2).

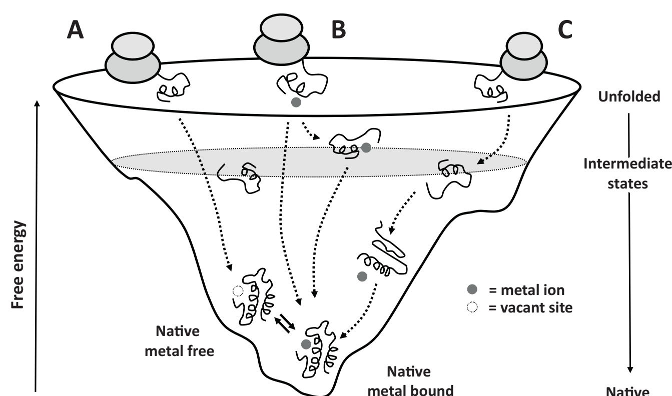

Fig. 2. Pathways for metal insertion into newly synthesized proteins. Protein synthesis carried out by ribosomes is overlaid on a folding funnel which depicts the energetic scale ranging from the unfolded conformations (top) with high energy and randomness, to the native folded state (bottom) corresponding to an energy minimum. Metals (or metal clusters) can be inserted into the polypeptide, either from transfer from a pool of free metal ions or *via* delivery by metallochaperones, through at least three distinct pathways: (A) after the polypeptide has adopted its final conformation and folded to an apo (metal free) state; (B) during translation before polypeptide release, which can result in the release of a metal-bound intermediate state or in the release of folded metal-bound proteins; and, (C) after release of a partially folded polypeptide to which metal binding further nucleates the folding process. Metal release from the native metal-bound form (holo) can result in either misfolding or unfolding as a result of conformational destabilization, or in a folded apo conformation with a vacant metal binding site.

Adapted from [9].

2. Protein misfolding and aggregation in the neuronal environment

Within the cellular environment, a number of factors influence protein folding, misfolding and stability, and metal ions are among key effectors. During protein biogenesis, the folding reaction of some proteins is catalyzed by molecular chaperones, a group of proteins that prevent the formation of non-specific interactions between incompletely folded chains and other macromolecules that would affect the formation of the native state. Molecular chaperones are part of the protein quality control (PQC) system which also comprises proteases and other folding catalysts (see [27] for a recent review). However, in spite of this tight control, protein misfolding may take place as a result of genetic (*e.g.* mutations) or cellular factors (*e.g.* oxidative stress, pH, macromolecular crowding, metal ions). Under these circumstances, the buildup of protein conformers which have a substantial number of its residues engaged in non-native interactions may lead to the formation of protein aggregates [28].

Protein pathologic aggregation is a common feature within neurodegenerative diseases such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD), among many others [29]. The fact that the majority of these neurodegenerative conditions are sporadic rather than genetic is suggestive that misfolding of the affected proteins may be determined by local environmental factors within neurons and the synaptic environment. In this section we will briefly describe the general nature of protein deposition processes and then focus into specific particularities of the chemical and physical setting of the synapse, discussing how it influences misfolding of proteins involved in neurodegenerative disorders.

2.1. Protein aggregation in neurodegeneration

Amyloid deposits are the hallmarks of many neurodegenerative diseases. The neuropathological basis of amyloid diseases builds on the 'amyloid cascade hypothesis', according to which the primary event in pathogenesis is the aggregation of the protein into soluble oligomers, and then insoluble fibrils. The complete process leading to deposition of highly organised mature fibrils proceeds through a considerable time frame. Under *in vitro* conditions mimicking physiological parameters this process may extend for months. However, the current view is that precursor non-fibrillar oligomers, which are formed from misfolded amyloidogenic species at the earliest stages of the protein deposition process are in fact highly cytotoxic and constitute the primary species contributing to disease [30]. In agreement, there is significant evidence showing that the presence of amyloid plaques does not correlate with disease severity [31,32].

In contrast to inert fibrils, oligomers are highly heterogeneous and structurally disorganized, enabling the exposure of amino acid side chain sequences to participate in aberrant interactions with other cellular components. Examples include (i) increased solvent accessibility of hydrophobic side chains that can promote

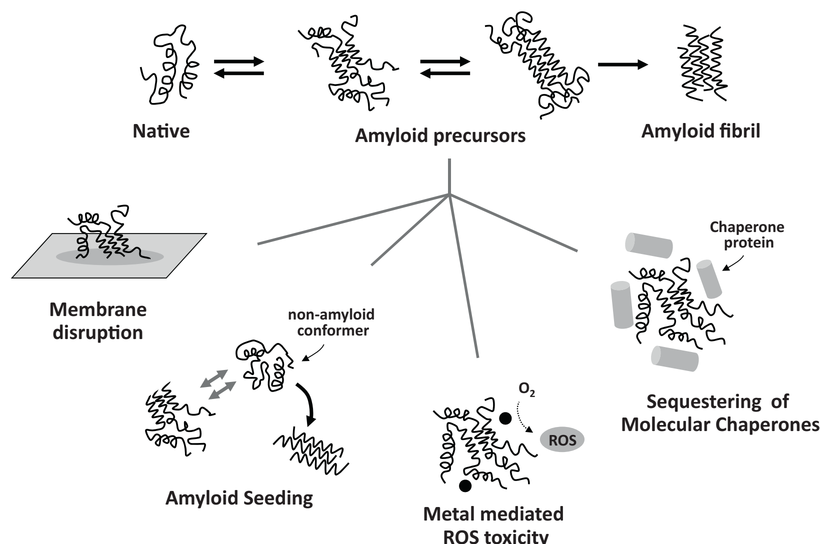

Fig. 3. Protein deposition into amyloid and toxicity pathways. Proteins and peptides undergo conformational changes due to environmental or genetic factors that result in the buildup of amyloidogenic conformers that will ultimately result into the formation of amyloid deposits *via* a series of precursor species which are currently known to be involved in toxicity pathways. These include disruptive interactions with biological membranes, seeding effects over non-amyloid conformers, metal ion (especially copper and iron) mediated ROS production and negative dominant effects such as those arising from sequestration of molecular chaperones.

protein–protein or protein–membrane interactions; (ii) exposure of amyloidogenic stretches with the potential to seed fibril formation by other proteins; (iii) aberrant metal binding sites that can either promote peptide cross-linking or the formation of reactive oxygen species (ROS) *via* Fenton reactions involving $Fe^{3+/2+}$ and/or $Cu^{2+/+}$ cycling, or; (iv) sequestration of folding chaperones that results in impaired monitoring of the protein quality control (Fig. 3). Cytotoxicity and neurodegeneration arises *via* a variety of combined cellular processes, including disruption of membrane integrity and tissue architecture, oxidative stress, mitochondrial dysfunction, chronic inflammation and impairment of cellular proteostasis, among other effects (*e.g.* [33,34]).

2.2. Influence of the synaptic milieu on protein deposition

Protein misfolding and deposition pathways in neurons are influenced by the physical and chemical properties of the synapse and its unique chemical biology, in spite of tight regulatory mechanisms including those of protein folding quality control [35]. Although we seek to emphasize the role of metal ions in neuronal dysfunction related to protein deposition, there is a close interdependence between multiple physical and chemical factors (*e.g.* macromolecular crowding, membrane interaction and physical confinement) that modulate the formation of proteinaceous aggregates in neurodegeneration. Thus, a brief general perspective of this complex crosstalk in the synaptic environment is useful.

Protein misfolding and deposition processes in neurodegeneration take place within the cytosol (*e.g.* tau deposition) and/or in the extracellular space (*e.g.* Aβ deposition) into the synaptic cleft, which is the gap between pre- and post-synaptic neuronal terminals. This region is further ensheathed by glial cells that actively regulate synaptic connectivity [36] (Fig. 4). There is an intense chemical and biological crosstalk in this confined space, typically about 20–40 nm wide [37], which is also highly crowded with macromolecules, metal ions and neurotransmitters. For example, there are about 1000 different proteins present in the human brain synapse [38], and a single synaptic vesicle releases on average 5000 neurotransmitter molecules [37]. By itself, this substantial macromolecular crowding effectively decreases solubility and promotes protein aggregation as a result of excluded volume effects that favor compact protein states [39]. In addition, the co-existence of large (polypeptides) and small (neurotransmitters) molecules near surfaces (cell membranes) results in entropy-driven depletion forces which effectively promote structural ordering [39]. This derives from the fact that upon protein-protein (or protein–membrane) interactions, the total solvent volume excluded from smaller crowders decreases, resulting in an effective attractive force between the large particles (*i.e.* proteins) or between the large particle and the surface (*i.e.* protein–membranes). This increases misfolding and aggregation propensity.

In fact, the large extent of membrane surface at the synapse is also a relevant modulator of the aggregation of amyloidogenic proteins when considering that these proteins have an extensive under-wrapping of backbone hydrogen bonds, which increases their propensity for protein-bilayer binding [40]. As an agreeing example, the interaction of the amyloid-β peptide (Aβ) with lipid membranes results in protein conformational changes and accelerated misfolding into toxic oligomers, which are enhanced by macromolecular crowding (*e.g.* [41]).

Labile metal ions are key factors in neurotransmission. Calcium influx following an action potential is fundamental for neurotransmitter release in neurons and is responsible for the large fluctuations of intracellular calcium concentration, from the nM (resting state) to the μM ranges (stimulated state) [42]. Zinc is concentrated into pre-synaptic vesicles of glutamatergic neurons in the labile ionic state and is co-released to the synaptic space with glutamate upon synaptic stimulation [43–45]. Free zinc concentrations may reach 60 μM in the glutamatergic synapse [46,47] and up to 300 μM in the mossy fiber boutons of neurons, whereas normal extracellular levels are 1–10 nM, as assessed from the cerebrospinal fluid (CSF). Copper concentration is also increased in the synaptic cleft during synaptic stimulation (up to 30 μM) and released post-synaptically upon activation of the NMDA receptor (NMDAR), which modulates the translocation of copper-containing vesicles to the synaptic cleft [42,46]. Altogether, this puts in evidence the high availability of labile metal ions within the neuronal environment.

The synaptic environment is also rich in proteins with metal-binding properties, many of which are present in the extracellular space (Fig. 4). These include proteins directly involved in the formation of toxic aggregates, such as the Aβ peptide, α-synuclein and

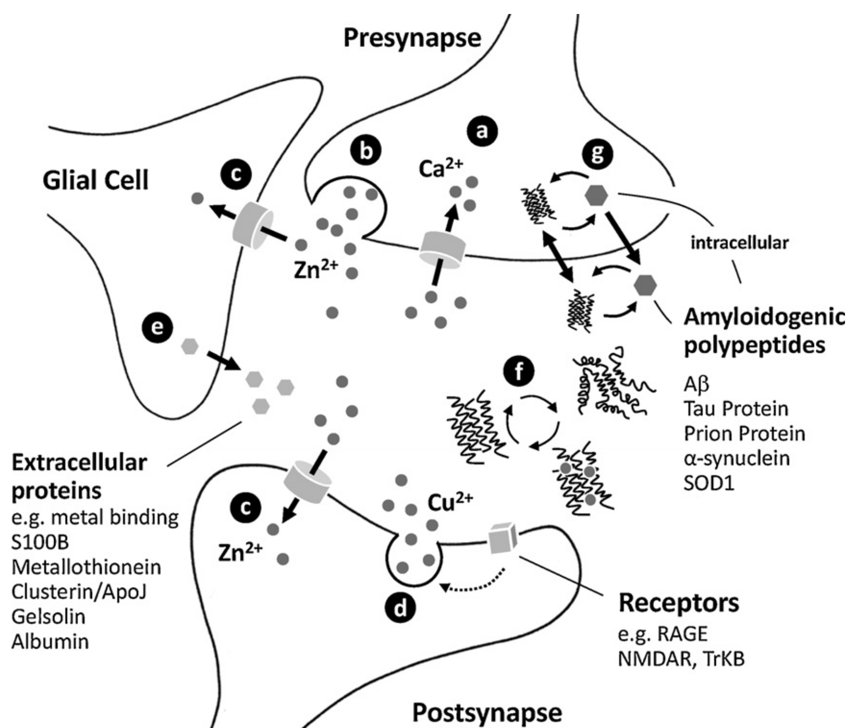

Fig. 4. Chemical and biological events involving metal ions and protein aggregates in the neuronal environment of the synaptic cleft. The cartoon depicts the neuronal environment and the synaptic cleft whose unique chemical and biological setting can promote protein deposition events. Metal ions are potential key players in this process as a result of the dynamic concentration fluctuation of labile metals during neuronal activity such as: (a) the influx of Ca^{2+} when the action potential reaches the neuron terminal; (b) Zn^{2+} release by synaptic vesicles at glutamatergic synapses and subsequent neuronal re-uptake (c) *via* different entry routes, such as calcium channels; and (d) post-synaptical Cu^{2+} release upon NMDAR activation. A considerable number of proteins are secreted into the synaptic cleft (e), many of which have metal binding properties that may mediate amyloidogenic interconversions of polypeptides (f). Proteinaceous deposits formed either intra or extracellularly have the possibility to migrate across membrane boundaries (g) and interact with different receptors that mediate a large number of cellular responses. See text for further details and references.

the prion protein, which have known metal binding properties and whose amyloid deposits in patients are substantially enriched in metal ions, such as is the case for zinc-loaded Aβ amyloid plaques.

However, other proteins with functional metal binding properties play also a secondary role in aggregation processes. These include proteins which are involved in metal buffering, minimization of Fenton reactions and ROS buildup such as metallothioneins (MTs); signaling molecules such as S100B—a calcium, zinc and copper binding protein, itself with amyloid forming propensity [48], which is secreted by astrocytes, accumulates in the synapse at high concentration and is overexpressed in AD; and folding chaperones such as clusterin/apoJ—an extracellular calcium-regulated protein with the ability to mediate the disposal of misfolded proteins and whose dysfunction has the potential to further aggravate misfolding [49,50].

Additionally, there is an intense cross-talk between protein deposition events taking place intra and extracellularly. The associated mechanisms range from interactions of amyloid conformers with membrane receptors such as RAGE and NMDAR that induce signaling cascades reshaping cellular responses, to trans-cellular signaling promoted either by intake of protein aggregates by surrounding cells or by intake of released metals during neurotransmission. Metal ions play a role in some of these processes: during intense synaptic activity, zinc accumulates in the synaptic cleft and enters postsynaptic spines, sharing entry routes with calcium [51]. Binding of Aβ oligomers to synaptic terminals is a process which can also be mediated by NMDAR and depends on a Zn^{2+} gradient [52]. This combination of factors makes the synapse an especially sensitive locus where complex interactions take place, many of which involve metal ions. The resulting dysfunction of neurons and glial cells in the synaptic environment further promotes misfolding *via* other mechanisms. For example, dysfunction of astrocytes in the tripartite synapses leads to a dysregulation of extracellular pH [36], a significant modulator of protein aggregation.

2.3. Calcium, copper and zinc dyshomeostasis in neurodegeneration

Unlike in any other organ, brain's specific physiology implies the coexistence of high levels of free metal ions within the synaptic cleft and neuronal cytoplasm. The homeostasis of these metal ions is the result of a fine balance between ion influx, sequestration, intracellular buffering and extrusion, that is crucial for safeguarding brain health and neuronal activity [53,54]. Any overload or deficiency in their cellular physiological concentration leads to a generally increased susceptibility of cells to undergo apoptosis [55–57], a common denominator in neurodegenerative pathologies. In this section we will briefly overview how defective homeostasis of calcium, copper and zinc impacts on different neurodegenerative conditions, in order to provide a cellular and physiological context for their effects on proteins. A number of excellent reviews are available for a more extensive coverage (*e.g.* [46,51,58–60]).

2.3.1. Calcium

Among neuronal metal ions, calcium dysregulation is likely the most susceptible to directly or indirectly result in neurodegeneration. Calcium is an ubiquitous intracellular signaling ion that in neurons functions also as a modulator of neurotransmitter release and excitability [61,62]. In order to attain signaling versatility cells maintain a tightly controlled resting concentration of cytosolic free calcium which is about 10^4 fold lower (≈50–100 nM) than the one found in the extracellular space (≈1–2 mM). In most neurons, intracellular homeostasis is challenged when synaptic transmission

takes place: action potential activation of nerve terminals triggers the opening of calcium channels leading to a profuse cellular influx of calcium ions, fundamental for neurotransmitter release. To be able to cope with this transient increase in free calcium concentration, neuronal cells rely on a complex homeostatic network made up of calcium-binding and buffering proteins for calcium extrusion and sequestration in intracellular stores (*e.g.* endoplasmic reticulum and mitochondria). Dysregulation of calcium homeostasis has been implicated in AD [60,63–65]. The calcium hypothesis for AD pathogenesis proposes that activation of the amyloid cascade by toxic forms of Aβ remodels neuronal calcium signaling pathways. By enhancing the entry of calcium and/or the release of intracellular calcium, neurons are rendered vulnerability to excitotoxicity and apoptosis. Calcium has also been implicated in PD pathology: cytoplasmic calcium concentrations are elevated in a particular set of dopaminergic neurons in the substantia nigra pars compacta (SNc) that establish the core symptoms of the disease [66]. Unlike most neurons, these SNc dopaminergic neurons generate an action potential in the absence of a synaptic input, exclusively through L-type calcium channels. Therefore, these channels are open most of the time yielding a constant calcium influx, implying unceasing calcium safeguard sequestrations with energetic costs. Sustaining this intensive calcium recycling activity relies heavily on mitochondrial activity, both for energetic production and incessant calcium buffering [67]. Due to the profuse entry of calcium in SNc dopaminergic neurons, this set of cells is suggested to be particularly vulnerable to calcium homeostatic stress, as well as metabolic stress, which are common features in PD [68,69]. Altered calcium homeostasis is also systematically found in patients that develop all forms of ALS, a fatal neurodegenerative disorder characterized by the specific degeneration of motor neurons. Interestingly, motor neurons are less able to cope with increased calcium levels due to a lack of calcium buffering proteins [70] along with intensive expression of calcium permeable glutamate receptors and ion channels [71]. These specific characteristics within motor neurons seem to converge and determine a selective vulnerability towards calcium dysregulation. In fact, calcium accumulates in motor neurons affected in sporadic [72] as well as in inherited ALS associated with mutations in the gene encoding SOD1 [73–75] and is proposed to be involved in neuronal death. The exact etiology of the disease is yet to be established and it is still not clear whether calcium dysregulation is the leading cause or a secondary pathological effect. However, it was reported that in cultured motor neurons expressing an ALS associated SOD1 mutant, the elevation of cytoplasmic calcium concentration favors SOD1 aggregation into inclusions and not *vice versa* [76]. This suggests that calcium plays a role in protein aggregation, which is a prominent hallmark of ALS. In fact, in most neurodegenerative diseases a positive feedback between calcium dyshomeostasis and disease-related protein aggregation has been reported [77].

2.3.2. Copper

Copper plays the primordial role of being the active redox centre in proteins that are involved in oxidase and oxygenase functions as well as in electron transfer and oxygen radical control. In contrast to this vital role, copper redox activity holds simultaneously the toxic potential to generate ROS and oxidative damage under reducing conditions. In order to avoid harmful side effects, cells have developed a complex system to ensure a fine homeostasis of copper levels. Upon entry into the cell, copper binds to a cascade of chaperone and accessory proteins that assure its correct targeting and transfer to specific compartments and client proteins, while avoiding its unspecific interaction with intracellular scavengers and uncontrolled harmful reactivity leading to ROS production. Any disruption in this network of copper trafficking and chaperoning proteins can lead to an imbalance of copper levels. In agreement, mutations/knock outs on copper transporters [78–80] result in disease. Apart from this highly specific copper regulatory network, metallothioneins (MTs) are unspecific metal ion scavengers that also assist the regulation of copper and zinc homeostasis, especially under cellular stress conditions [81,82]. In fact, MTs are primarily expressed within the brain where they are suggested to function as backup copper storage proteins [83]. In spite of these chelating mechanisms, copper dyshomeostasis occurs in many neurodegenerative disorders like AD and Huntington's disease (HD), where it is known to exacerbate neurodegeneration, as recently reviewed [58]. Copper accumulation generates neurotoxicity not only by promoting protein aggregation [44,84–86] but also as a result of ROS production. For example over-expression of several genes involved in copper metabolism in a yeast model of HD and overexpression of MTs in mammalian cells significantly reduced huntingtin aggregation and toxicity [85].

2.3.3. Zinc

Most intracellular zinc is essentially found in a tight protein-bound form where it is involved in multiple biological processes as an essential cofactor for protein folding, conformational changes or biological activity. In its labile form, zinc modulates neurotransmission and synaptic function and regulates many signaling pathways. Thus, the level of free cytosolic zinc has to be strictly regulated in order to maintain its homeostasis, as both zinc overload and deficiency induce susceptibility to apoptosis [55,56,87–94]. Zinc homeostasis is essentially regulated by the combined activity of zinc transporters (ZnTs), zinc-importing proteins (ZIPs) and by the buffering action of proteins such as MTs, as well as sequestration systems (*e.g.* mitochondria) that maintain intracellular free levels within the picomolar range. Whenever any of these systems is impaired, the resulting accumulation of zinc affects primarily mitochondria, disrupting the cellular energy system and trigging ROS production, with subsequent oxidative damage. In the central nervous system (CNS), zinc is particularly susceptible to accumulate in a class of glutamatergic neurons found almost exclusively in the neo-cortex and limbic structures. In this set of neurons, free zinc is stored at high levels (≈1 mM) in presynaptic vesicles and co-released with the neurotransmitter. Thus, during intense synaptic activity, zinc can accumulate in the synaptic cleft and profusely re-enter adjacent cells, sharing entry routes with calcium. Interestingly, abnormally high concentrations of free zinc are observed in the neo-cortex of AD patients where Aβ amyloid deposits are selectively formed, despite the ubiquitous expression of Aβ throughout the brain [95]. This suggests that the amyloid neuropathology of AD could derive from synaptic zinc release during glutamatergic neurotransmission events. In agreement, knocking out the synaptic ZnT3 zinc transporter in the Tg2576 AD mouse model resulted in no synaptic zinc accumulation and in a marked decrease in cerebral Aβ deposition [96]. Zinc is also dysregulated in ALS and suggested to play a key role in pathological processes associated with this disease [97]. MT expression is markedly up regulated in the brain and spinal cord of ALS patients, as well as in ALS mutant SOD1 transgenic mice [98–102] where accumulation of labile zinc in neurons has been reported [103]. Deletion of MTs in a mutant SOD1 transgenic mouse model results in accelerated symptom onset and shorter survival time [104,105]. This is consistent with MTs having a protective role by buffering toxic free zinc. Zinc dyshomeostasis is also present in PD, evidenced by a significantly decreased level of zinc in the CSF [106] and significantly increased levels in the substantia nigra [107], as well as increased MT expression [108]. Overall, zinc dyshomeostasis in AD, ALS and PD disorders is concomitant with mitochondrial dysfunction and downstream oxidative damage [109–111]. In a feed-forward cyclic manner, increased levels of labile zinc lead to mitochondrial dysfunction that triggers ROS generation and cellular oxidation,

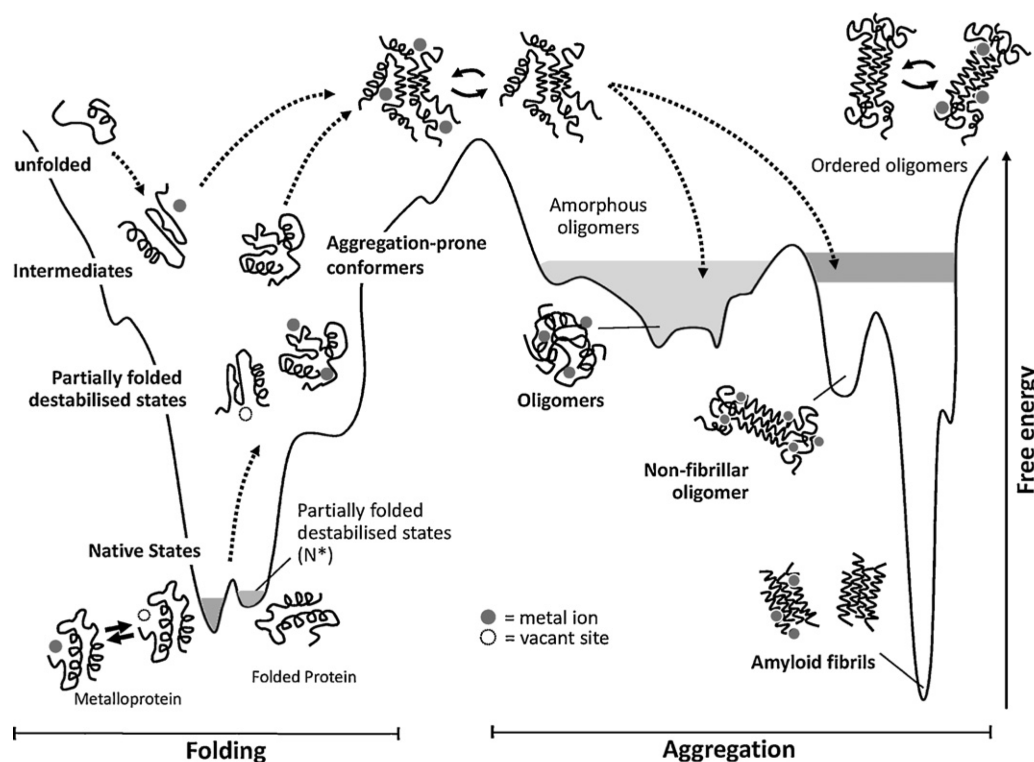

Fig. 5. Generic scheme depicting mechanisms of metal-mediated protein deposition. Conceptual cartoon depicting multiple pathways for the effects of metal ions in the formation of protein deposits represented in the context of the energetic landscape of folding and aggregation funnels. Metal ion interactions with folding intermediates, with destabilized apo or folded states, or with amyloidogenic conformers may result in the formation of amyloidogenic aggregates and precursors. The pool of aggregates which becomes accessible to these species includes that of amorphous aggregates and ordered oligomers, the latter leading to amyloid fibrils or to off pathway non-fibrillar oligomers. See text for further details and references.

which, in turn, promotes further zinc release, thus perpetuating pathology.

3. Effects of metalation on proteins involved in neurodegeneration

Having established (i) how metals influence protein conformations and folding, (ii) that metal ions are essential in brain neurobiology, and (iii) that an imbalance on their homeostasis is strictly related to neurodegeneration, in this section we will overview how metallation impacts on proteins involved in aggregation diseases.

3.1. Mechanisms of metal-mediated protein deposition

The protein aggregation landscape comprises a set of non-native conformations such as amorphous aggregates, oligomers and amyloid fibrils. Since metal ions affect protein dynamics, folding and stability, it is clear that metal binding/release can also have an important role in protein aggregation. Indeed, metal ions can specifically dictate protein misfolding establishing a bias towards accelerated fibrillization or amorphous conformations (Fig. 5).

3.1.1. Metal induced misfolding: pathways to aggregation

Most of the proteins involved in neurodegenerative disorders associated with protein deposition are intrinsically disordered. The high structural dynamics of these proteins and the broad landscape of accessible conformations accounts for the reason why metal binding is such a powerful modulator of protein aggregation. In fact, metal binding may promote aggregation either by inducing the folding or stabilization of distinct aggregation-prone conformers. There are abundant examples of these effects which will be further discussed into following sections. For instance, binding of copper and iron to α-synuclein leads to the formation of molten-globule type conformers which results in an increased aggregation rate. One other example is that of zinc binding to the prion protein (PrP), which shifts the aggregation pathway towards the formation of amorphous aggregates instead of fibrils. Metal to protein ratios also determine the type of modulating effects: in the case of Aβ, high concentrations Zn^{2+} and Cu^{2+} account for the formation of cytotoxic amorphous aggregates whereas equimolar concentrations promote fibrillization. SOD1 aggregation provides a contrasting example as the mature metallated from of this metalloenzyme is structured and very stable. In this case, it is the absence of proper metal binding that populates native-like destabilized conformers which are prone to aggregate.

3.1.2. Metal binding to oligomers

Metal ions can also act at the level of intermolecular interactions critical for the formation of multimeric aggregates. This will result in cross-linking reactions involving a remodeling of the aggregation landscape by triggering interconversions between distinct types of oligomers. Indeed, metal binding to oligomeric Aβ also takes place, although at lower affinity. This can however result in increased toxicity if the bound metal is able to mediate ROS production. By itself this promotes further aggregation by inducing protein oxidative modifications. Interestingly, there may even be selective binding of certain metal ions to specific oligomeric conformers. That is the case of calcium and magnesium, which bind specifically to paired helical filaments but not to the native tau protein. The consequence of this selective binding is that it shifts tau aggregation pathway towards amorphous oligomers instead of neurofibrillary tangles.

Table 2
Properties of proteins forming aggregates in neurodegenerative conditions.

	α-Synuclein	Tau	SOD1	Prion	Amyloid-β peptide
Disease	Parkinson's disease	Alzheimer's disease	Amyotrophic lateral sclerosis	Transmissible spongiform encephalopathies	Alzheimer's disease
Inclusions	Lewy bodies; Lewy neurites	Neurofibrillary tangles	Intraneuronal inclusions	Amyloid plaques	Senile plaques
Expression[†]	Neurons presynaptic terminals	Neurons	Ubiquitous	Neurons	Neurons
Proposed function	Unclear function: neuronal plasticity, neuroprotection against oxidative stress and excitotoxicity	Neurogenesis, induction of tubulin assembly, microtubule stability	Antioxidant; scavenging of superoxide anion	Unclear function: neuroprotection; copper homeostasis; antioxidant; apoptosis inhibitor; calcium signaling	Unclear function: neurotrophic; scavenger of redox active metal ions
Major cell localization	Cytosolic, membrane bound, some extracellular	Cytosolic, membrane bound	Cytosolic, mitochondrial intermembrane space	Extracellular plasma membrane	Extracellular some intracellular
Protein structure	Soluble: disordered monomer and α-helical tetramer. Membrane bound: α-helical	Disordered	β-Sheeted structure Homodimer	PrP^C: cellular form is a monomeric α-helical glycoprotein with an unstructured domain. PrP^{Sc}: misfolded scrapie isoform is a β-sheet rich oligomer	Disordered
Metal binding affinities[a]	Cu^{2+} (0.1–280 nM)[b] Fe^{2+} (10 μM) Ca^{2+} (2–300 μM)	Cu^{2+} (0.5 μM) Zn^{2+} (3.8 μM) Cd^{2+} (320 μM) Hg^{2+} (0.1 nM)	Cu^{2+} (6 fM)[c] Zn^{2+} (1.8–10 nM)	Cu^{2+} (0.1 nM to 12 μM)[d] Zn^{2+} (98 nM–200 μM) Ni^{2+} (16 nM) Mn^{2+} (202 μM)	Cu^{2+} (0.1–60 nM)[e] Zn^{2+} (1–300 μM)
Metals modulating aggregation	Ca^{2+}, Cu^{2+}, Fe^{3+}, Al^{3+}, Zn^{2+}, Co^{2+}, Mn^{2+}	Fe^{3+}, Al^{3+}, Zn^{2+}, Cd^{2+}, Ca^{2+}, Mg^{2+}	n.d.	Mn^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}	Ca^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+}, Al^{3+}

[†] type of cells where the expression of the corresponding protein is more abundant.
[a] Range of affinity constants (K_d) reported in the literature are indicated between parenthesis.
[b] Additional very low affinity copper sites have been reported [138,144].
[c] As determined for yeast SOD1 [401].
[d] Range as reported for the four octarepeat sites; two additional low affinity copper binding sites are found outside of this region [287,289].
[e] A very low affinity site has also been reported [363].
n.d. not determined. See text for references.

3.2. *Metal ions and protein deposition processes in neurodegeneration*

Having established general mechanisms through which metal binding affects protein (mis)folding and aggregation pathways, we will here systematize particular aspects of the role of metals in key proteins in paradigmatic neurodegenerative conditions (Table 2).

3.2.1. *α-Synuclein*

Several genetic, biochemical and pathological data implicate α-synuclein in the onset and development of several neurodegenerative diseases, including PD, AD, dementia with Lewy bodies, Lewy body variant of Alzheimer's disease, multiple system atrophy, collectively called synucleinopathies [112–115]. The most common synucleinopathy is PD, where pathology is tightly associated with α-synuclein deposition in the form of intracellular amyloid deposits called Lewy bodies, in dopaminergic neurons in the substantia nigra [116]. α-Synuclein is an abundant neuronal protein, enriched in presynaptic terminals [117–119]. It is mainly cytosolic although membrane-bound and extracellular forms have been identified [120–124]. The soluble α-synuclein monomer is recognized as an IDP [125–128], however, recent studies indicate that it can occur physiologically as a helically folded tetramer [129,130], although it remains unclear whether or not this is the main physiological form, considering observations showing that the protein expressed in the nervous system exists predominantly as an unfolded monomer [131].

3.2.1.1. Metal binding to α-synuclein. α-Synuclein binds multiple physiological and non-physiological metal ions, most of which interact with α-synuclein unspecifically [132,133]. α-Synuclein binds Cu^{2+} at high affinity, although the binding stoichiometry, affinity and ligands are a matter of debate. Two thermodynamically distinct binding modes have been proposed for the consensual N-terminal high affinity site around neutral pH [134,135], accounting for dissociation constants in the 0.1–0.4 nM [135,136] or ∼100 nM ranges [137]. The binding site is composed by N-terminal amino acid residues (N-terminal amine, Asp-2, Val-3) [134,136]. The involvement of the imidazole ring of His-50 in Cu^{2+} coordination is a matter of intense debate [136–139]. The reason for the conflicting results may be the exchange of His-50 and a water molecule for binding to Cu^{2+} at pH 7.4 proposed by Davies et al. [135]. It has been proposed that upon Cu^{2+} binding to this site α-synuclein acquires a locally more structured conformation which is thought to drive aggregation [140]. A second Cu^{2+} site of unknown identity and lower affinity has also been reported [136,141,142] (K_d ∼36 μM, [143]). α-Synuclein is also able to bind Cu^{2+} unspecifically, most likely at the acidic C-terminus. This allows the protein to accommodate a maximum of 16 Cu^{2+} ions [138,144]. In addition, there are reports for two Fe^{3+} binding sites (K_d = 10 μM) [145] and for a Ca^{2+} binding site located at the C-terminal acidic 32-amino acid domain (K_d = 2–300 μM) [146].

3.2.1.2. Metals and α-synuclein aggregation. Multiple data suggest an involvement of metal ions in α-synuclein aggregation: (i) environmental exposure to iron, copper, manganese, lead, zinc and aluminum is associated with increased Parkinson's prevalence [147]; (ii) iron, zinc and aluminum accumulate in the substantia nigra of Parkinson's patients, when compared with controls [107,148–150]; and (iii) metal ions modulate the aggregation

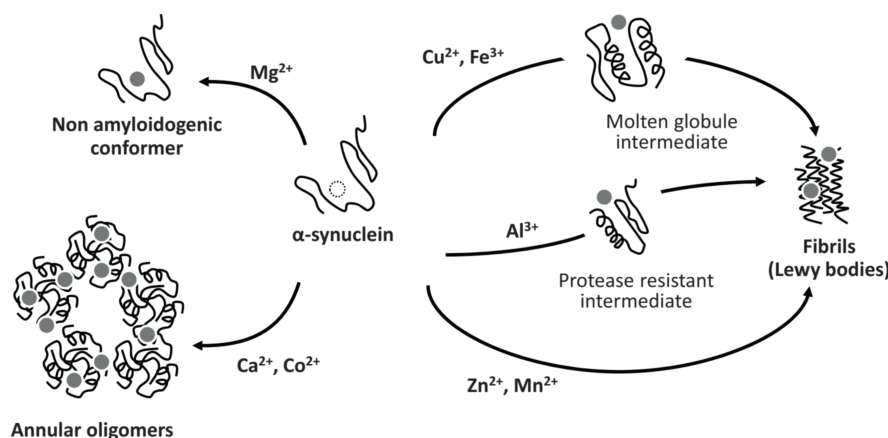

Fig. 6. Metal ions in α-synuclein conformation and aggregation. α-synuclein is a protein forming amyloid Lewy body deposits in a range of synucleinopathies. Specific or unspecific metal binding (*e.g. via* chronic environmental exposure) modulates α-synuclein aggregation pathways. Cu^{2+} and Fe^{3+} bind specifically and enhance the formation of amyloid fibrils involving the formation of a molten globule-like intermediate. Zn^{2+} and Mn^{2+} binding facilitate fibril formation, while Al^{3+} is the most effective metal ion in accelerating fibrillization, *via* the formation of a structured, proteolysis-resistant intermediate. Other metal ions trigger non amyloidogenic aggregation processes: Ca^{2+} and Co^{2+} complexes form annular oligomers and Mg^{2+} has anti-amyloidogenic properties. See text for further details and references.

and fibrillization of α-synuclein *in vitro* (Fig. 6). Indeed binding of Cu^{2+} and Fe^{3+} to α-synuclein enhances the fibrillization rate [132] by facilitating the formation of partially folded, molten globule-like intermediates [140]. Nevertheless, the formed fibrils have distinct morphologies. On the one hand, Cu^{2+} promotes the formation of β-like structures in monomeric α-synuclein [151] as well as thin, long and interwoven fibrils [152]. On the other hand, Fe^{3+} promotes the generation of short and thick fibrils [152] and at low micromolar concentrations induces stable oligomers, on pathway to fibrils [153] which are toxic *in vitro* and *in vivo* [132,154,155]. Reactive species arising from copper binding oxidize α-synuclein's methionine residues and generate metal-oxidized protein oligomers [156–158]. The fact that Zn^{2+} reverts methionine oxidized oligomers into fibrils [159] may be a relevant factor for *in vivo* processes. In addition, Zn^{2+} also promotes α-synuclein fibrillization *in vitro* [132,160,161]. Ca^{2+} promotes α-synuclein aggregation *in vitro* and *in vivo* [162] and is suggested to be a critical factor in boosting α-synuclein aggregation in PD by enhancing the protein–membrane interaction [133]. Indeed, incubation of α-synuclein with Ca^{2+} results in the formation of annular oligomers [163], similar to the ones which have been extracted from the brains of multiple system atrophy patients [164,165]. In sharp contradiction to its non-physiological nature, Al^{3+} is the strongest enhancer of α-synuclein fibrillization [132]. Al^{3+} induces a conformational change in α-synuclein rendering a more structured [132,166], protease-resistant conformation [167]. At high protein concentration (>100 μM), the structured Al^{3+}–α-synuclein complexes spontaneously self-associate and promptly convert to fibrils [132]. Also, manganese intoxication *per se* recapitulates many PD symptoms [168,169]. Mn^{2+} binding increases the secondary structure content of α-synuclein and enhances fibrillization [132]. In accordance, simultaneous α-synuclein transfection and Mn^{2+} incubation significantly reduced cell viability [170]. Finally, Mg^{2+} has an opposite action, inhibiting α-synuclein aggregation [171] and outcompeting the action of Al^{3+}, Mn^{2+}, Cu^{2+} and Zn^{2+} [172]. These results could elicit a neuroprotective role for Mg^{2+} on substantia nigra's dopaminergic neurons [173].

3.2.2. Tau protein

Tau is a disordered protein [40,41] whose abnormal aggregation occurs in AD, among other tauopathies [174]. It is preferentially expressed in neurons as a cytosolic protein, but is also associated with membranes [175,176]. It plays a role in neurogenesis, inducing tubulin assembly and promoting microtubule stability [177,178]. Tau deposition is triggered by hyperphosphorylation that abolishes the otherwise stabilizing interaction of tau and microtubules, resulting in free disordered tau protein that assembles into intracellular paired helical filaments (PHF) (Fig. 7). Nevertheless soluble oligomers that precede PHF assembly [179] are in fact proposed to be the cytotoxic species [180–182]. Ultimately these aggregates assemble in neurofibrillary tangles (NFT) in the amygdala, hippocampus, entorhinal cortex, association cortex and sensory cortex [183].

3.2.2.1. Metal binding and tau aggregation. Metal binding to tau is almost exclusively associated with misfolding and initiation of amorphous aggregation or fibrillization. Tau binds Al^{3+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+} [184–189] and, with the exception of Cu^{2+}, all ions have an effect in tau aggregation (Fig. 7) [184,188,189]. Binding of Fe^{3+} and Al^{3+} is favored by tau phosphorylation and is likely mediated by metal–phosphate electrostatic interactions [190,191]. Al^{3+} binding has been studied intensively because this metal ion lacks biological function but induces the formation of large amorphous aggregates [192–194] and selectively accumulates within NFTs *in vivo* [195]. Fe^{3+} also binds to hyperphosphorylated tau and induces its aggregation [188,189], essentially into PHF conformers [189], while reduction to Fe^{2+} can reverse aggregation [189]. Interestingly, Ca^{2+} and Mg^{2+} bind to PHF but not to phosphorylated tau, resulting in amorphous aggregates [196]. NFT harboring neurons accumulate excess iron [195,197], which catalyzes Fenton reactions *in situ* [197,198], generating oxidative stress and likely perpetuating tau hyperphosphorylation [199]. Although Cu^{2+} does not have a significant impact on tau aggregation *in vitro* [184], it binds to tau with micromolar affinity (∼0.5 μM) and induces α-helical structuring of the tau monomer, a characteristic of PHFs [184,185,200]. Interestingly, binding of Zn^{2+}, Cd^{2+} and Hg^{2+} to tau has been recently described and shown to induce faster fibrillization [186]; also, metallation impacts on aggregation kinetics, which correlates directly with metal binding affinity ($Hg^{2+} > Cd^{2+} > Zn^{2+}$). The effect of Zn^{2+} is particularly interesting: at low micromolar concentrations it accelerates tau fibril formation whereas at higher concentrations granular aggregates are formed [201].

3.2.3. Superoxide dismutase 1

SOD1 is an abundant and ubiquitous antioxidant enzyme [202,203] that is expressed essentially in the cytosol [204], although it is also found in the mitochondrial intermembrane space [205,206]. More than 100 point mutations scattered throughout the

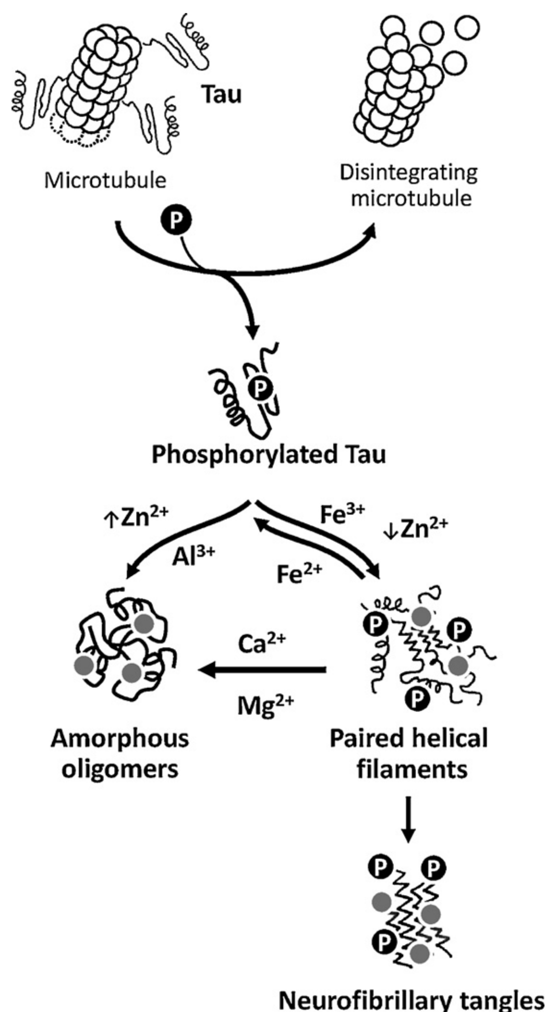

Fig. 7. Modulation of tau aggregation by metal ions. Tau protein hyperphosphorylation abolishes the otherwise stabilizing interaction of tau and microtubules. In the phosphorylated (P) state, tau undergoes amyloidogenesis, assembling into paired helical filaments and, ultimately, amyloid fibrils in intracellular neurofibrillary tangles. Tau phosphorylation also facilitates the binding of Fe^{3+} and Cu^{2+}. Cu^{2+} does not promote fibrillization itself but rather the acquisition of α-helical content similar to that of PHFs. On the other hand, Fe^{3+} promotes tau fibrillization. The reduction of Fe^{3+} to Fe^{2+} reverts PHF formation and is also involved in the generation of reactive oxygen species *in situ*, which may be relevant to perpetuate hyperphosphorylation. On the other hand, Al^{3+} induces the formation of amorphous tau oligomers, off the amyloidogenesis pathway.

SOD1 sequence cause a subset of amyotrophic lateral sclerosis (ALS) [207,208], a fatal neurodegenerative disease that targets and progressively degenerates motor neurons. Pathology is not based on the loss of SOD1 enzymatic activity [209–212]. Instead, it is proposed that familial ALS etiology must depend on the gain of toxic properties by mutated SOD1. Human patients with SOD1 linked ALS indeed have cytoplasmic proteinaceous inclusions in the spinal cord enriched in SOD1 aggregates [213–219]. These occur early in disease and increase as pathology progresses, exclusively in the affected neuronal tissue [210,220]; thence it has been proposed that SOD1 linked ALS is a protein misfolding disorder where the particular physiology of the neural tissue is also likely to play a key role in the onset of SOD1 aggregation [221].

3.2.3.1. SOD1 structure and folding. SOD1 is a very stable homodimer that holds a disulfide bridge as well as a functional copper and zinc binuclear site in each monomer. Each chain folds into a β barrel that is flanked by two major loops: the zinc and the electrostatic loops, which together shape the active site. Copper associates directly to the β-sheet scaffold, and its insertion is mediated by CCS, a specialized chaperone acting in the intra mitochondrial space. Zinc binds to the zinc loop and the intra-subunit disulfide links this loop to the β barrel [222–224]. The metal binding organization of Cu/Zn site in SOD1 is quite singular. Both metal ions share residue His-63 as a common bridging ligand *via* the imidazolate ring. Cu^{2+} is coordinated by His-46, His-48 and His-120, in a distorted square planar arrangement whereas Zn^{2+} is coordinated by Asp-83, His-71 and His-80, in a tetrahedral geometry. In the absence of copper and zinc binding, the β barrel and dimer interface remain intact, but the major loops have a high level of disorder [225–227].

3.2.3.2. Metallation status and aggregation propensity. SOD1 typifies a case in which metal ion binding is crucial for folding and maintenance of the native conformation. In this case, there are so far no reported effects of metal induced SOD1 aggregation.

In the absence of metals SOD1 is severely destabilized yielding conformers with a high propensity to aggregate at physiological pH, regardless of the disulfide's redox state [228–231]. This vulnerability of the apo state to self-association is related with the high flexibility and disorder of the zinc and electrostatic loops [232,233]. Indeed, zinc plays a major stabilizing role as its coordination alone – without copper binding – modulates the loops' native organization [234–237], yielding SOD1 the necessary 'negative design' that results from well-ordered loops and dimerization which protect the β-strand edges from self-association. Zn^{2+} binding is also necessary to pre-organize copper ligands prior to Cu^{2+} coordination [226,236,238]. In fact, SOD1 maturation in the cell is thought to begin with the coordination of zinc into the reduced monomeric state, as only then is CCS able to simultaneously load the catalytic copper cofactor and oxidize the intra-subunit disulfide bond [239] (Fig. 8). However, the mechanisms through which zinc is loaded into SOD1 remain elusive [240].

Mis-metallation can also trigger SOD1 toxic deposition. In fact, zinc has a promiscuous behavior towards SOD1 metal binding sites, being able to jump between the copper and zinc ligands [234]. At the early stages of SOD1 folding, Zn^{2+} binds to the copper site accelerating the folding reaction. As the reaction progresses and the zinc binding site, which structures late in the folding process, is organized Zn^{2+} is transferred to its most thermodynamically favorable condition at the high affinity zinc site [241]. If the zinc ligands are mutated, copper ligands can initially coordinate the non-native zinc ion at μM affinity; however, without the structural support of the loaded zinc site the protein misfolds and has an increased propensity to aggregate [238]. Moreover, zinc is found at both copper and zinc binding sites in recombinantly expressed SOD1. Double zinc binding results in significant changes at the copper site when compared with the properly metallated form, but coordination stability is identical [242]. Altogether, zinc dynamics and promiscuous coordination are critical to the folding of native SOD1, a key aspect as the majority of ALS patients with SOD1 inclusions do not carry any mutation on this gene [243–247]. Interestingly, in the familial cases, a common feature of SOD1 pathological mutations is an increased aggregation propensity relatively to the wild type [248,249]. Also, mutant SOD1 aggregates tend to be metal-deficient and/or lack the disulfide bond, raising the possibility that the disease causing mutations may increase levels of SOD1 intermediate conformers (Fig. 8). In agreement, the folding pathway of SOD1 pathological mutants has been suggested to favor the accumulation of metal depleted monomeric intermediates [250], the most effortless state for formation of aggregates [231,251].

3.2.4. Prion protein

The cellular prion protein (PrP^C) is an ubiquitous [252] cell surface glycoprotein conserved in mammals. It is preferentially

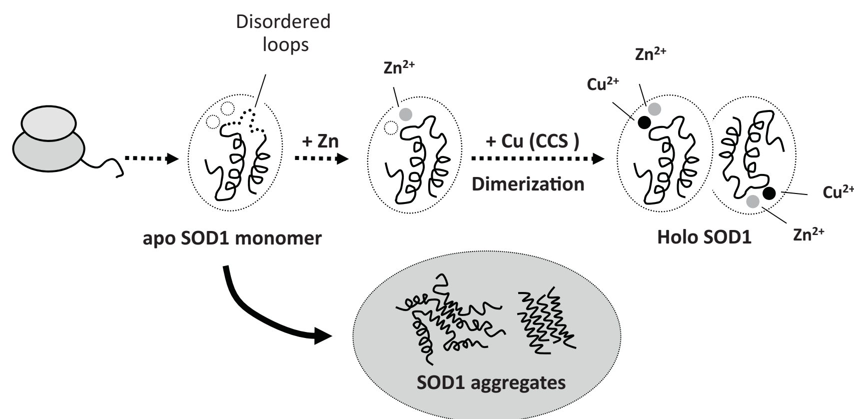

Fig. 8. Biogenesis of SOD1, metal ion insertion and aggregation prone conformers. The newly synthesized SOD1 monomer folds into a native-like apo conformation, which comprises disordered loops, and is prone to aggregate. Zinc insertion organizes the connecting loops and subsequent insertion of copper *via* the CCS machinery yields the formation of the intrasubunit disulfide and dimerization. See text for further details and references.

expressed at the CNS [253,254] within synaptic membranes [255–257]. The function of PrP^C is unclear. However, since the identification of its Cu^{2+} binding properties [258], numerous studies have implicated PrP^C in copper homeostasis, antioxidant protection and calcium signaling [257,259,260]. The unique neuropathology of the prion protein stems from the discovery that the propagation of transmissible spongiform encephalopathies (TSEs) such as Creutzfeldt–Jakob disease (CJD) [261,262] is mediated solely by a proteinaceous infectious particle (*i.e.* a 'prion') [263]: the misfolded (or scrapie) form of the prion protein (PrP^{SC}) [264,265].

3.2.4.1. PrP structure. Mature PrP^C is a two domain protein, comprising an unstructured N-terminal region (residues 23–120) and a α-helical rich C-terminal domain (residues 121–231) [266–268]. The N-terminal domain features PHGGGWGQ octarepeats (four in the case of the human protein) responsible for high affinity metal binding (residues 60–91), flanked by two positively charged clusters (residues 23–27 and 95–110). A hydrophobic region (residues 111–134) connects the N-terminal and the C-terminal domains. The C-terminal domain (residues 121–231) is rich in α-helices (∼40% of all protein) with almost no β-sheet content (∼3%) [266,268–270]. Additionally, there is a single intermolecular disulfide bridging residues 179 and 214 in helices 2 and 3 [267,268,270], two glycosylation sites at Asn-181 and Asn-197 [271,272] and the GPI binding site at Ser-230 [273] for membrane tethering [274,275]. PrP^C is monomeric [266–268] and protease sensitive [276]. On the other hand, PrP^{SC} is a β-sheet rich (30% α-helix and 43% β-sheet [269]), oligomeric and proteolytic resistant conformation [276] which self-templates its propagation [276] leading to amyloid plaque deposition [277] and progressive vacuolation of brain tissue [278].

Copper binding to the prion protein occurs *in vivo* at physiological CSF Cu^{2+} concentrations and its binding is strongly preferred over other metal ions [46,258,279]. PrPC can bind between 1 and 4 Cu^{2+} ions *in vivo* while retaining its soluble form [280] (Fig. 9). Cu^{2+} binding to the octarepeat regions, especially at full saturation, has been computed to promote structuring, compactation and establishment of long range contacts of the otherwise unstructured N-terminus [281]. Altogether, this suggests a function as a copper binding protein. Up to 1 Cu^{2+} ion can bind per octarepeat [282,283], as well as 1 other ion to His-96 and His-111 [283–287]. Conflicting evidence has been presented regarding the existence of additional Cu^{2+} binding sites in the C-terminal folded domain of PrP^C [287–293], which do not seem to involve histidine side chains [287] as previously proposed by experimental [288,289] and theoretical analysis [291]. The first Cu^{2+} ion binds exclusively to three or four imidazole side from histidine residues within an octarepeat (K_d ∼0.1 nM) [294]. When 2 Cu^{2+} ions bind, each one is coordinated by ligands from two adjacent octarepeats [294]: histidine imidazole and backbone nitrogen of one octarepeat are the equatorial ligands together with two water molecules whereas the histidine imidazole from the other octarepeat provides axial ligation (K_d ∼12 μM) [294]. At a 4:1 ratio, each octarepeat binds one ion equatorially coordinated through the histidine imidazole and backbone nitrogens and carbonyls in the HGGGW sub-sequence and axially by a water molecule (K_d ∼7 μM) [282,295]. Only at higher stoichiometries does Cu^{2+} bind to the extra-octarepeat histidines His-96 and His-111 [287]. Other metals, such as Zn^{2+}, Fe^{2+} and Mn^{2+}, also bind to PrP, although with lower affinity [293,296]. From these, only Zn^{2+} coordination has been thoroughly assessed. This also occurs *via* one or two octarepeat histidine side chains [293,297,298], but saturation is achieved after only 1 Zn^{2+} equivalent is bound. Zn^{2+} may be a natural PrP^C ligand [298,299] *in vivo*,

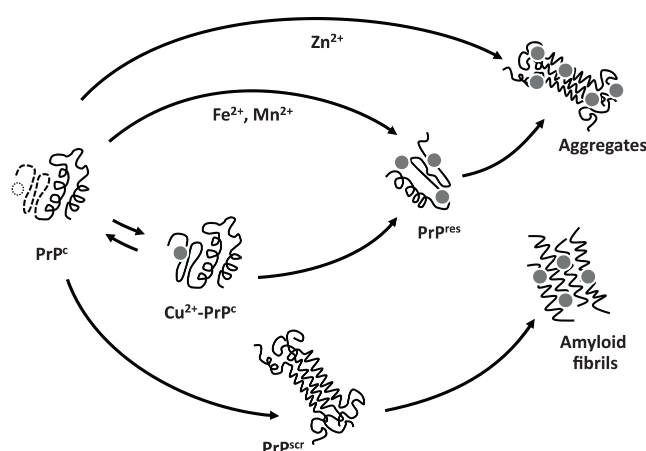

Fig. 9. Conformational transitions of the human prion protein upon metal binding. The cellular prion protein (PrP^c) can undergo spontaneous misfolding into a β-sheet rich conformation (the scrapie prion isoform, PrP^{sc}) which templates misfolding of further native proteins and drives amyloid polymerization. The physiological function of PrP^C is thought to be associated with reversible Cu^{2+} binding (between 2 and 4 Cu^{2+} ions). However, in the presence of Fe^{2+}, Mn^{2+} or higher concentrations of Cu^{2+}, PrP^C undergoes a conformational change to a β-sheet rich and protease-resistant conformation (PrP^{res}), which ultimately forms amorphous aggregates. In the case of Zn^{2+} binding, no intermediate has been detected before the formation of the amorphous aggregates. See text for further details and references.

owing to its larger synaptic concentration [300]. Due to ligand competition, Zn^{2+} is able to change the Cu^{2+}–PrP^C coordination mode, without displacing Cu^{2+} [293]. Brown et al. have proposed that PrP^C binds Mn^{2+} by replacing Cu^{2+} but have also admitted that the final conformation is aberrant [301], in line with the report by Garnett and Viles suggesting that Mn^{2+} does not bind to the octarepeat region.

3.2.4.2. Metals and PrP aggregation. Unlike in other proteins undergoing aggregation in neurodegenerative diseases, metal ions modulate the self-association of PrP exclusively promoting non-amyloidogenic toxic aggregates (Fig. 9). Upon Cu^{2+} binding, PrP^C undergoes a conformational transition yielding a conformer which is highly protease resistant, has enhanced β-sheet content [302] and is neurotoxic [303]. This species, from here on referred to as PrP^{res}, inhibits fibrillization and leads to the precipitation of large protease-resistant aggregates [304–307]. In this case, a locally misfolded conformer wherein a His-96–Cu^{2+}-His-111 cross-link drives the formation of a β-hairpin involving Pro-102 and Pro-105 at the turn region has been observed in molecular dynamics simulations and may represent the initiator for misfolding and aggregation [308,309]. Cu^{2+} also binds to pre-formed prion fibrils, rendering protection against proteolytic degradation [304]. The relevance of Cu^{2+} in triggering prion aggregation is highlighted by the fact that copper chelation delays the onset of disease in PrP^{SC} infected mice [310]. One can envisage that the specific local folding induced by tight Cu^{2+}–PrP^C binding, which affects mainly the unstructured N-terminal domain, prevents the establishment of the β-sheet core of amyloid and is responsible for the selection of the non-amyloid aggregation pathway [311].

Binding of Mn^{2+} induces the PrP^{res} conformation [296,301], although binding is slower than for Cu^{2+} [312]. This conformational transition ultimately leads to prion aggregation [313,314], which occurs at 100 times lower metal concentration than that of Cu^{2+} [299]. Interestingly, abnormally elevated Mn^{2+} levels are present in the blood and CNS of CJD patients [315]. Mn^{2+}–PrP is only toxic to cells when endogenous PrP is expressed, suggesting a seeding effect towards endogenous PrP^C [316]. Conversely, Mn^{2+}-specific chelation therapy in animals infected with prion disease was effective in extending their lifespan [301].

Similar to Cu^{2+}, Zn^{2+} is inhibitory of PrP^C fibrillization [304], can bridge prion molecules [317] and produces neurotoxic aggregates [303]. Zn^{2+} controls the aggregation of the prion protein similarly to Cu^{2+} as both shares the four octarepeat histidine ligation, when bound 1:1 to PrP^C [293]. Lastly, Fe^{2+} binding to PrP [299,301,318], induces the formation of the PrP^{res} state [296] where the iron-associated oxidative stress is increased [319,320]. Quantum mechanical studies suggest that Fe^{2+} specific effects may be based on a substantial deformation of the octarepeat, which does not occur on Cu^{2+}, Zn^{2+} or Mn^{2+} binding [321].

3.2.5. Amyloid-β peptide

The Aβ peptide corresponds to a 39–43 amino acid long fragment of the amyloid precursor protein (APP), generated by β- and γ-secretase proteolytic processing [322]. Although APP is expressed in many tissues, highest expression occurs in the brain [323,324]. When secreted in the monomeric intrinsically unstructured state, Aβ is thought to exert neurotrophic functions [325–328], although a neuroprotective copper scavenging role has also been suggested [329]. The deposition of Aβ in the form of extracellular amyloid plaques in the brain cortex and hippocampus [330] is a characteristic hallmark of Alzheimer's disease (AD), the most common form of dementia. The most abundant Aβ isoforms are the 40 ($A\beta_{1-40}$) and 42 ($A\beta_{1-42}$) amino acid long variants. $A\beta_{1-42}$ is produced in lower amounts but is more amyloidogenic and thus comprises most of the Aβ pool in plaques [331]. At neutral pH, $A\beta_{1-40}$ and $A\beta_{1-42}$ are thermodynamically soluble up to 15.5 μM [332–334] or 2 μM [332], respectively, in sharp contrast to the low nanomolar Aβ concentrations found in the CSF of normal subjects and AD patients [333,335–339]. Oligomeric species produced during Aβ fibrillization – but not fibrils [340], are cytotoxic and result in AD neurodegeneration [52,341–348].

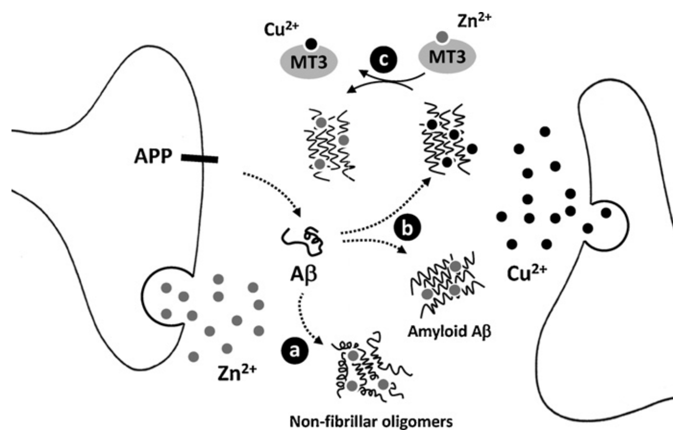

Fig. 10. Aβ deposition and metal ions in the synaptic cleft. The Aβ peptide is secreted into the synaptic cleft upon APP proteolytic processing, where it undergoes amyloidogenesis processes in Alzheimer's disease. Metal ions released into the synaptic space during neurotransmission – such as Cu^{2+} and Zn^{2+}, see Section 2.3 – can modulate the peptide's amyloidogenic pathways. High concentrations of Zn^{2+} and Cu^{2+} are responsible for the formation of cytotoxic Aβ amorphous aggregates (a). On the other hand, both Zn^{2+} and Cu^{2+} equimolar concentrations drive Aβ fibrillization (b). The generation of ROS by Cu^{2+}-Aβ fibrils can be alleviated by MT3-dependent metal swap with zinc (c). See text for further details and references.

3.2.5.1. Metal binding to amyloid-β. Metal ions constitute one of the most important modulators of Aβ folding and aggregation landscapes (Fig. 10). Aβ binds zinc, copper, calcium, iron and aluminum [349–353] and these metal ions accumulate in senile plaques [354–356]. Much of the knowledge of the binding of metal ions to Aβ has been gathered using peptides representing the N-terminus of Aβ, which contain all the ligands and are not required for fibril formation [357,358]. The binding of metal ions to Aβ invariably results in aggregation, which may either be of the amorphous or amyloid type, depending on the metal ion and stoichiometry. Zn^{2+} binds to Aβ stoichiometrically (1:1) with a broad affinity range from 1 to 300 μM [64–66]. Binding takes place through N-terminal residues: the side chains of His-6, His-13, His-14 in combination with the N-terminal amine, Asp-1 or Glu-11 carboxylates, water or hydroxide [359–362]. Aβ can bind up to two copper ions in the N-terminus around physiological pH through histidine side chains and other N-terminal residues (reviewed recently in [359,363]). A tentative explanation to the difficulty in the identification of a specific set of Cu^{2+} ligands in Aβ has been recently proposed by Ali-Torres et al. [364] in a combined homology modeling and quantum mechanics study which computed several energetically close three nitrogen one oxygen Cu^{2+}-Aβ adducts where the specific coordination sphere is the outcome of a synergic relationship between the peptide and metal. However, contrary to metalloproteins, the Cu^{2+} binding sites are not preformed [363]. The first Cu^{2+} ion binds with a conditional affinity constant which is not fully consensual, ranging from 0.1–1 nM [359] to 30–60 nM [363]. The binding of Cu^{2+} to the second Cu^{2+} site occurs with ∼100 times lower affinity [365,366], making it unlikely that this binding site is populated under physiological Cu^{2+} concentrations [365].

3.2.5.2. Metals and Aβ aggregation. The regulation of Aβ aggregation and amyloidogenesis by Zn^{2+} is complex. Upon binding,

Zn^{2+} induces a conformational change wherein the Aβ N-terminus wraps around the Zn^{2+} ion exposing a mostly hydrophobic surface [367], which may drive self-association. Transient micromolar zinc bursts, akin to synaptic signaling pulses, accelerate $Aβ_{1–40}$ misfolding and aggregation and stabilize toxic oligomers at physiological pH *in vitro* [368,369] and *in vivo* [52,329]. Contrary to the Zn^{2+} pulses, steady state equimolar Zn^{2+} concentrations produce non-toxic amorphous $Aβ_{1–42}$ aggregates [333,370,371] which slowly convert to fibrils [372]. The delay was proposed to be due to the depletion of apo fibrillization prone Aβ [370]. Zn^{2+} is incorporated in mature fibrils with similar affinity to the soluble form [373], recapitulating the Zn^{2+}-rich composition of senile plaques. If higher than equimolar concentrations are used, fibrillization is inhibited through the formation of amorphous aggregates [352,374–376]. It has been proposed that Aβ aggregates *via* the formation of intermolecular His-Zn^{2+}-His cross-links [377], the same ligation mode observed in *ex vivo* plaques [378]. Zn^{2+} may also specifically bind fibril ends and function as a cap preventing further elongation [379].

Copper binding also promotes aggregation. In the presence of sub-stoichiometric Cu^{2+}, $Aβ_{1–42}$ assembles into amyloid fibrils [368,380,381]; however, at higher stoichiometries, $Aβ_{1–40}$ and $Aβ_{1–42}$ form 10–20 nm wide spherical oligomers and large insoluble cytotoxic amorphous aggregates [366,370,376,380,382] which are unable to seed fibrillization of monomeric $Aβ_{1–42}$ [380]. Nevertheless, in spite of the distinct morphologies the aggregation kinetics is not significantly changed at these different ratios [366]. The conformational changes taking place upon metallation of the Aβ species have been investigated by a number of theoretical and computational approaches. For instance, it has been proposed that Cu^{2+} binding induced amorphous aggregation results from distortion of the Aβ amyloid fibril backbone [383]. Cu^{2+} binding was proposed to bring together the 1–10 and 11–16 stretches of Aβ [384], and the consequent ordering of this N-terminal stretch is a factor driving amorphous aggregation, as this region has been computed to be unstructured in Aβ fibrils [357]. Still, these reports only account for the amorphous Aβ aggregation occurring at 2:1 Cu^{2+}-Aβ stoichiometry.

There is an interesting crosstalk between Zn^{2+} and Cu^{2+} in what concerns effects on Aβ aggregation. Similarly to Zn^{2+}, a fibril inhibition effect is also observed for Cu^{2+}, but only at three times higher concentrations [370]. The proposed mechanism suggests a depletion of the aggregation competent monomeric apo Aβ [370]. On the other hand, due to the similar binding sites, Zn^{2+} can replace Cu^{2+} in a metallothionein dependent mechanism [385,386], which is deficient in the AD brain [387,388]. This metal swapping yields redox-inert species thus minimizing ROS-mediated toxicity [389].

Ca^{2+} binding to Aβ also leads to the formation of non fibrillar oligomers [351]. These were proposed to further perturb membrane integrity and Ca^{2+} transport within cells, generating a Ca^{2+} dyshomeostasis positive feedback loop contributing to disease progression. Fe^{3+} is thought to have a minor role in Aβ aggregation processes because it associates poorly with Aβ *in vivo* [390,391], when compared with zinc and copper. However, Fe^{3+} binds Aβ at high affinity, accumulates in senile plaques (up to ∼1 mM) [197,354,392,393] and delays the formation of mature fibrils [394]. This determines the accumulation of smaller fibrils and unstructured aggregates which may contribute to toxicity [352,394].

Finally, the effects of Al^{3+} binding to Aβ have gathered much attention. As observed for other metals, Al^{3+} binds to the N-terminus of Aβ [18], delays fibrillization and specifically promotes the formation of highly cytotoxic oligomers [18,353,395–397] that can be reverted upon Al^{3+} chelation [398]. Interestingly, apart from accumulating in senile plaques it enhances Aβ secretion [399], which is indicative of a possible role of Al^{3+} in AD.

4. Outlook

Labile metal ions such as calcium, zinc and copper are vital in neuronal biology. Yet, these very same metals can potentially modulate toxic protein deposition in several neurodegenerative conditions. The physiological features of certain neurons are certainly relevant in determining why these proteinaceous deposits are formed predominantly in specific cells of the nervous system. For example, the fact that Aβ deposition occurs mainly in glutamatergic neurons in which zinc bursts transiently result in high synaptic concentrations of this metal ion is likely a relevant factor in AD etiology. The role of metal ions as modulators of protein deposition and neurotoxicity thus arises from the intersection of biological processes and the metallochemistry of specific neurons.

The specific physicochemical properties of polypeptides also determine why certain proteins have selective propensity to undergo metal ion dependent aggregation. In fact, most of the protagonist proteins engaged in neurodegenerative disorders are intrinsically disordered, or stage some level of intrinsic disorder. Their underlying structural flexibility, combined with promiscuous metal binding and affinity within physiologic concentrations in neurons, is thus a factor favoring aberrant metal–protein interactions.

Upon binding, metal ions largely influence the aggregation of these proteins, either by promoting folding into aggregation prone conformers or by stabilizing non-amyloidogenic conformations, which have increased toxicity. From the considerable number of studies here overviewed, it is clear that metal ions can determine multiple protein deposition processes and promote the formation of a multitude of protein aggregates. These broadly range from toxic soluble oligomers to precursor fibrils and to inert amyloid deposits. An even deeper understanding of these phenomena at the chemical and biological levels will be essential for future pharmacological interventions aimed at preventing these processes. From the clear role played by metals in protein aggregation processes, the so-called metal targeted strategies aimed at remodeling of the brain metal redistribution rather than strict chelation are among future possibilities for future therapeutic developments [400].

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