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Metal ions and amyloid fiber formation in neurodegenerative diseases.

Copper, Zinc and Iron in Alzheimer's, Parkinson's and Prion disease

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Running Head: **Metal ions and amyloid fiber formation**

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Abbreviations: Aβ Amyloid-β-peptide; AD, Alzheimer's disease; CD, Circular Dichroism; N-methyl-D-aspartate receptors, NMDAR; Parkinson's disease, PD; Prion protein, PrP; α-Synuclein, αSyn; ThT, Thioflavin T; transmissible spongiform encephalopathies, TSEs; TEM, Transmission Electron Microscopy.

Abstract

There are a group of diseases associated with protein misfolding and accumulation into amyloid fibers. Many of these diseases have a major impact on human health, in particular, Alzheimer's (AD), Parkinson's (PD) and Prion diseases. The focus of this review is to highlight how metal ions influence amyloid formation in a number of neurodegenerative diseases. Firstly, the various mechanisms by which metal ions might influence the kinetics of amyloid fiber formation are surveyed. The coordination of metal ions to a number of amyloidogenic proteins, with an emphasis on metal binding to intact fibers is reviewed. The kinetics of amyloid formation and the influence Cu^{2+}, Zn^{2+}, Fe^{3+} and Ca^{2+} have on amyloid-beta peptide (Aβ) fiber formation in AD is described in detail. The effect of metal ions on fibril formation for other amyloidogenic proteins, in particular Cu^{2+} binding to α-synuclein (αSyn) and the prion protein (PrP), are also reviewed. The mechanism by which metal ions might influence neurotoxicity of amyloids is also discussed. Levels of metal ions found at the synapse are described and related to the affinity of metal ions for Aβ, PrP and αSyn. I*n vivo* evidence for a link between metal ions in these common neurodegenerative diseases, and the interplay between Aβ the prion protein and copper are reported. Finally, the possibility of a shared mechanism by which metal ions might influence amyloidosis is discussed.

Contents

1) Protein Misfolding Diseases

Central to a number of neurodegenerative diseases and other diseases of protein accumulation and amyloidosis is the misfolding of individual proteins [*1*]. In Alzheimer's disease (AD), the most common form of dementia, a small ~42 residue peptide, amyloid-beta peptide (Aβ), is cleaved from a larger amyloid precursor protein (APP). It is the accumulation of the Aβ molecules into toxic oligomers and amyloid fibers that appears fundamental to the cascade of events, including the formation of neurofibrillary tangles of tau protein, central to the aetiology of AD dementia [*2*]. Similarly, in Parkinson's Disease (PD) the natively unstructured protein, α-Synuclein (αSyn), accumulates into β-sheet rich fibers within intra-cellular inclusion bodies, known as Lewy Bodies [*3*]. While misfolding of the mammalian prion protein (PrP) causes a group of transmissible spongiform encephalopathies (TSEs) in humans and other mammals [*4*]. The misfolding of the proteins triggers a cascade of events in these diseases, often including the accumulation of ordered fiber aggregates of these proteins, with amyloid structure rich in β-sheet [*1*]. Genetic alterations underlying familial forms of these diseases are associated with mutations or increased production of Aβ, PrP and αSyn, indicating that these proteins play a central role in their respective diseases [*3-5*]. All three of these diseases have been linked to metal ion binding and changes in metal homeostasis [*6, 7*]. Other protein misfolding diseases will be briefly discussed within this review because of their association with metal ions.

2) How can metal ions induce protein misfolding and accumulation?

The influence of metal ions on protein misfolding does not require an external environmental influence, simply a perturbation in metal ion homeostasis and compartmentalization [*6-9*]. The Cu^{2+} and Zn^{2+} ions are found concentrated within senile plaques of Alzheimer's disease patients directly bound to Aβ [*10-13*]. In PD, elevated levels of copper and iron ions have been reported in the cerebrospinal-fluid and Lewy bodies respectively [*14, 15*]. In the case of the prion protein (PrP^{C}), Cu^{2+} ions are known to bind to *in vivo* and *in vitro* [*16, 17*] and influences prion protein levels in the brain [*18*]. Metal imbalance is an early charcteristic of prion disease [*19*] and Cu^{2+} has been found in scrapie isolates (PrP^{Sc}) and confers prion strain type [*20*].

Concentration of these metal ions in amyloid fibers raises the possibility that these ions might trigger or promote amyloid formation. There are a number of ways the coordination of metal ions can influence fiber generation. These possible processes are illustrated in Figure 1. For example, the coordination might cause rearrangement of the protein main-chain and so trigger misfolding and subsequent protein accumulation, Figure 1. Cu^{2+} binding to β_2-microglobulin

(β_2M) has been shown to cause a key proline cis-trans isomerization, triggering misfolding and protein assembly in dialysis-related amyloidosis [*21*]. Coordination of the metal ion might involve inter-molecular cross-linking, Figure 1. Formation of dimers or high order oligomeric forms may again influence the protein misfolding and accumulation. For example, *in vitro* Zn^{2+} can form an inter-molecular complex with Aβ, cross-linking between histidine residues on multiple Aβ molecules, that inhibits fibrillization [*22-24*]. Coordination of a metal ion will typically affect the net charge of the protein, adding positive charge from the metal ion or losing charge from multiple deprotonation. As a consequence, a protein with an acidic pI may become more neutrally charged and therefore more prone to self-association. This mechanism is proposed to accelerate fiber formation kinetics when Cu^{2+} binds to Aβ [*23*]. Amyloid fibers are in equilibrium with monomeric and oligomeric forms; it is possible that the metal ions might stabilize the fibril or oligomers once formed, by for example, cross-linking via the metal ion. Alternatively, coordination of the metal ion could destabilize the normal non-pathogenic structure and so make misfolding more energetically favorable, Figure 1. This has been shown to occur when Cu^{2+} binds to the normal cellular form of the prion protein [*25*]. The fibril morphology might also be influenced by metal ions. Fe^{3+} ions have been shown to influence the morphology and toxicity of Aβ fibers [*26*] while Cu^{2+} influences prion strain type [*20*].

Furthermore, coordination of redox active metal ions, such as copper and iron might also influence protein accumulation by metal catalyzed chemical modification of the protein. For example, reactive oxygen species (ROS) have been shown to generate an Aβ dimer by covalent cross-linking of tyrosine residues within Aβ [*27*]. Oxidation of the methionine and histidine residues have been reported for both Aβ and PrP [*28, 29*]. The majority of the methionine in Aβ (Met35) is oxidized within amyloid plaques [*10*]. While a large proportion of isolated scrapie (misfolded) prion protein is known to contain methionine residues oxidized to methionine-sulphoxide [*30*]. The chemical modification will in turn influence the stability, fiber forming kinetics and structure of the protein.

3) The relationship between fibers, oligomers and neurotoxicity

Initially mature amyloid fibers found in AD patients were identified as the neurotoxic entity in AD pathology, this concept was modified when it was found that small diffusible oligomers of Aβ, rather than mature amyloid fibers were the more toxic form [*31-33*]. However a role for fibers in Aβ neurotoxicity should not be ignored, as there remains strong evidence suggesting amyloid plaques, or possibly intermediates of the Aβ fibrils, play a critical role [*34, 35*]. Small

oligomers rather than mature fibrils have also been highlighted as the most neourotoxic form for a number of other misfolding diseases [*36*]. Protein oligomers may be precursors to fiber formation but may also arise from fiber fragmentation [*37*]. Studying oligomers is extremely challenging as they tend to form heterogeneous mixtures and are often transient in nature. The toxic oligomers may share the same structural features as larger fibers, the increased toxicity may simply arise from an increase in the number of toxic elements per total protein mass [*37*]. A range of oligomeric forms have been identified with various structural features and oligomer sizes, which can be classified into pre-fibrilar oligomers, fibrillar oligomers and anular protofibrils using their antibody binding properties [*33*]. These oligomers can be on or off the pathway to fibers, in addition not all oligomers are cytotoxic. Both fibers and oligomers require the self-association of protein, thus factors that affect fibrillisation will also influence oligomer generation. Furthermore, amorphous aggregates, oligomers and fibers are all in equilibrium with the monomeric protein, thus stabilization or destabilization of one will perturb levels of the other. The equilibrium set-up between different forms is illustrated in Figure 2.

Although it may be that oligomers are the most toxic to cells, it appears that the rate of fibril formation *in vitro* can be strongly correlated with rates of disease progression in patients and animal models of the disease. This may be because conditions that promote amyloid formation also promote oligomers. For example, the familial early onset AD, associated with Aβ mutations (E22K/G/Q) show considerably accelerated fibril growth times relative to the wild-type sequence [*38*]. Furthermore, the disease associated Aβ(1-42) is much more prone to rapidly forming fibers than Aβ(1-40). Similarly, mutations in αSyn associated with familial PD cause accelerated kinetics of fiber formation [*39*].

Despite significant progress in identifying misfolding proteins as central to the aetiology of a number of neurodegentrative diseases, the mechanism by which misfolded proteins are toxic is still not well established. The toxic action of may be intracellular, disrupting the mitochondrial membrane for example [*32*]. However, one popular hypothesis involves oligomers and fibers acting on the outer plasma membrane surface to disrupt membrane integrity [*32*]. The growing ends of fibers with exposed hydrophobic residues may be the region that perturbs membrane integrity. Furthermore, reactive oxygen species (ROS) generated at the membrane can cause lipid per-oxidation to further compromise the membrane [*40*]. The enhanced cytotoxic effects of Cu^{2+} bound to Aβ might be explained by concentrating redox active Cu^{2+} ions at the plasma membrane surface where they will generate harmful ROS [*23, 41*]. A recent study has

highlighted the role for PrP^C in mediating Aβ neuronal toxicity and describes how copper influences this process [*42*]; for more details see section 10.3.

4) Amyloid fiber structure and metal ion coordination

Amyloid fibers are typically un-branched fibers 7-12 nm thick and many microns long with a high proportion of β-sheet structure. The beta strands stack perpendicular to the long axis of the fiber forming intermolecular hydrogen bonds, this type of structural motif is known as a cross-beta structure. A range of biophysical methods have been applied to studying the structure of fibers. They include, X-ray fiber diffraction, solid-state NMR, cryo-EM, deuterium exchange and EPR of spin labels. The biophysical approaches and structural features of various amyloids have been reviewed [*43-45*]. The morphology of fibers and consequently their pathology can vary depending on the conditions in which the fibers form, metal ions will affect the morphology or 'strain' of fibers [*20, 26*].

The coordination of metal ions to a number of amyloidogenic proteins have been studied extensively. For recent reviews in the area of Aβ, see [*46-49*], for the prion protein [*50, 51*] and for αSyn [*52*].

4.1) Cu^{2+}-Aβ structure: Cu^{2+} binding to Aβ shows that one mole equivalent of Cu^{2+} bound to monomeric or mature Aβ fibers have identical coordination geometries [*53, 54*] and affinities [*53*]. The β-pleated core of Aβ fibers occurs between residues 14 to 40, Cu^{2+} coordinating ligands falls just on the edge of this region. Solid-state NMR of the Cu^{2+} complex suggests that the fibrillar structure is not disrupted by Cu^{2+} coordination [*55*], figure 3. The Aβ peptide contains three histidine residues (His^6, His^{13}, His^{14}), which along with the N-terminal amino group and aspartate form a tetragonal complex with Cu^{2+} ions [*53, 54, 56-64*]. A considerable amount of effort has been devoted to understanding the coordination geometry of Cu^{2+} bound to Aβ and is the subject of a number of reviews [*46-48*]. Some of the details of the complex are yet to be agreed upon, and the model shown in Figure 3 [*53*] is only one of a number related structures suggested in the recent literature. A dynamic view of the Cu^{2+} complex involving imidazole coordination in both the axial and equatorial plain has now emerged [*53, 57, 64, 65*]. A numbers of interchangeable Cu^{2+} complexes from with related coordination geometries and different histidine sidechains in the equatorial plain and pH effecting their relative distribution [*53, 57, 64, 65*].

4.2) Cu-PrP structure: Less well understood is Cu^{2+} binding to the fibrillar form of PrP. Models of recombinant PrP amyloid fibers suggest that the core residues forming β-strands within PrP are between residues 160-225 [*66, 67*]. Thus in amyloid fibers the Cu^{2+} binding N-terminal residues (23-126) remain unstructured and may well bind Cu^{2+} with a similar coordination geometry as the cellular mammalian PrP, but this remains to be established.

Metal binding, Cu^{2+} in particular, has been studied extensively for the cellular monomeric prion protein, PrP^C. As many as six Cu^{2+} ions coordinate to the natively unstructured N-terminal half of mammalian PrP^C at physiological pH. The structured C-terminal domain of PrP^C does not affect Cu^{2+} coordination, as its removal does not affect binding to the natively unstructured N-terminal domain, PrP(23-126) [*16*]. Cu^{2+} loads onto full-length PrP^C sequentially; the first equivalents of Cu^{2+} bind to the amyloidogenic region of PrP^C, residues 90-126, followed by binding to the octarepeat region, residues 58-91 [*16*]. There are various binding modes, all involve tetragonal coordination to one or more histidine imidazole nitrogens.

PrP contains a repeating motif of eight amino acids, between residues 58 and 91, with each repeat containing a histidine residue. This highly conserved region binds up to four Cu^{2+} ions with identical coordination geometry [*68, 69*], Figure 3. A crystal structure of the Cu^{2+} bound octarepeat motif (residues HGGGW) indicates square-pyramidal geometry [*70*] involving coordinating nitrogen and oxygen ligands from the main-chain as well as the imidazole sidechain. A more complete picture of the modes of Cu^{2+} coordination shows that at sub-stoichiometric levels, Cu^{2+} ions will bind with a higher affinity to the octarepeats via multiple histidine residues [*71-74*], Figure 3.

Cu^{2+} ions binding outside the octarepeat region are centered at His^{95} and His^{110} (His^{96} and His^{111} in the human sequence) [*75-84*]. A square-planar/tetragonal complex is formed upon Cu^{2+} binding, which involves the histidine imidazole nitrogen δN and the main-chain amides that precede the histidine [*79, 80*], Figure 3. This type of complex is similar to that in the octarepeats but is more stable because it involves a six membered chelate ring to the imidazole nitrogen rather than seven, Figure 3. There is reasonable agreement in the literature regarding the Cu^{2+}-PrP binding modes. The coordination geometries shown in Fig 3b may be a simplification of the coordination modes, as with the Cu-Aβ complex a number of related complexes may form interchangeably and their relative abundance will depend on the pH and levels of Cu^{2+}. Indeed sub-stoichometric Cu^{2+} and lower pH values favor multiple histidine sidechain coordination [*16, 71-74, 80*]. Affinity measurements place the K_ds, at pH 7.4, for

Cu^{2+} binding in the amyloidogenic region and octarepeats (multiple histidine binding mode) at 30 nM, tighter than Cu^{2+} binding to individual octa-repeats (90 nM) [*85*]. Although, others have reported tighter binding in the multiple His binding mode [*71-73*], suggesting Cu^{2+} will bind here first rather than concomitantly with Cu^{2+} binding centered at His^{110} [*16*].

4.3) Cu-αSyn structure: αSyn is largely found in presynaptic terminals in the reducing environment of the csytosol, here αSyn will experience Cu^{+}. There have been some studies of Cu^{+} coordination to αSyn [*86*]. However, a proportion of αSyn is found extra-cellularly, secreted by neuronal cells [*87, 88*], and so Cu^{2+} binding may also be relevant and has been studied more extensively. The coordination geometry and affinity of Cu^{2+} ions to αSyn has been characterized by a number of groups. A range of spectroscopies including absorption, visible-CD and EPR has been used to characterize the coordination geometry [*89*]. While the paramagnetic broadening effects of Cu^{2+} has been studied using solution NMR [*90*]. Redox properties and coordination of the synuclein family of proteins have also been described [*91, 92*]. EPR and pulsed EPR methods have been used to carefully characterize the Cu^{2+} coordination to full-length natively unstructured αSyn [*93, 94*]. It seems clear the main locus of binding to αSyn is at the N-terminal amino group. At pH 7.4, two related tetragonal complexes dominate, with almost equal affinity. Both modes of coordination involve the N-terminal amino-group and the amide main-chain nitrogen and carboxylate side coordination from aspartate (Asp) at position 2. Mode 1 is indicative of 2N2O ligands (Mode 1:NH_3, N_{amide} $C_\beta COO$, and water) while Mode 2 is more indicative of 3N1O ligands and may possibly contain an imidazole nitrogen His51 to form a macrochelate to replace water coordination (Mode 2: NH_3, N_{amide} , $C_\beta COO$, His_{imd}) [*93-95*]. Recently a related Cu^{2+} complex is described at pH 6.5 [*96*]. The affinity for αSyn is 0.1-0.4 nanomolar [*94, 97, 98*] while other metal ions have weaker mM affinities for αSyn [*99*]. It is notable that the formation of amyloid fibers of αSyn may not restrict Cu^{2+} coordination centered at the N-terminal amino group.

4.4) Common feature of Cu^{2+} and Zn^{2+} coordination: A feature of all three of these Cu^{2+} binding proteins is that the binding region is natively unstructured, thus the coordination site is not preformed but there is a structural rearrangement as the Cu^{2+} binds to the protein. All involve at least one imidazole nitrogen to form a tetragonal complex. A feature of PrP and αSyn is main-chain amide coordination to the Cu^{2+}, which makes these binding sites quite specific to Cu^{2+} ions as few other metal ions are capable of amide deprotonation [*79, 80, 100*]. Zn^{2+} binding to proteins does not typically mimic the Cu^{2+} coordinating ligands. In particular,

Zn^{2+} is not able to coordinate amide main-chain nitrogen. In addition, Zn^{2+} ions form tetrahedral complexes rather than tetragonal. There is evidence of Zn^{2+} causing inter-molecular cross-linking of imidazoles within Aβ molecules and multiple His imidazole coordination from the octarepeats of PrP [*22, 46, 63, 101*].

5) Kinetics of fiber formation:

5.1) Nucleation-dependant polymerization:

The kinetics of amyloid fiber formation are often described as a nucleation-dependant polymerization reaction [*1*]. This process involves the protein monomer forming a nucleating 'seed', this is then followed by a more rapid self-templated growth where the ends of existing fibers recruit protein monomer and so extend fiber length. There is still debate as to the precise nature of the minimal form of protein that nucleates fiber formation. Suggestions range from misfolded monomeric species, dimer, penta to larger oligomers.

In vitro, the kinetics of fiber formation typically follows a sigmoidal fiber growth curve, Figure 4 [*44, 102*]. Key observables include the lag-phase (nucleation) and the maximal growth rate (elongation). Fiber growth then plateaus, reaching equilibrium with low amounts of monomer, known as the critical concentration of fiber formation. A key feature of the nucleation-dependant polymerization reaction, is the ability of small amounts of pre-formed fibrils to 'seed' the reaction reducing the lag-phase significantly, Figure 4.

It is well established *in vitro* that kinetics of fiber formation are also often strongly influenced by agitation or sonication, which will cause significant fragmentation of fibers. It is now clear that a secondary nucleation process, in particular fragmentation, can significantly reduce the observable lag-time. The fragmentation-assisted growth has the effect of self-seeding fibril formation and can dominate the kinetics of fibril growth [*103*].

5.2) Monitoring Fiber Kinetics:

There are various methods by which the kinetics of fiber formation can be monitored *in-vitro [102]* . These include measuring a change in structure as β-sheets form within the fiber (via, for example, CD) or change in particle size via dynamic light scattering. A common approach is to use fiber specific dyes. In particular, Thioflavin T (ThT) which when bound to amyloid fibers fluoresces at 487 nm [*102*]. This fluorescence signal is directly related to the amount of amyloid present. The kinetics of fiber growth are very sensitive to a number of factors such as pH, concentration, agitation, temperature and ionic strength, these must be carefully controlled

in vitro for direct comparisons. Solubilisation of the protein or peptide into a seed-free form is also important. Typically, a fluorescence well-plate reader is used for the ThT measurements so that repeat fiber growth measurements can be made under identical conditions for a direct comparison.

5.3) Growth Curve Analysis:

The fiber growth curve, Figure 4, can provide a number of empirical parameters, these including lag-time (t_{lag}), the apparent rate of elongation (k_{app}) and the time taken to reach half maximal fibre intensity (t_{50}). The fiber growth curve can be fitted to the following equation [*104*].

$$Y = (y_i + m_i x) + \frac{(\upsilon_f + m_f x)}{(1 + \exp^{-(X-Xo/\tau)})} \qquad \text{(eq 1)}$$

Where Y is the fluorescence intensity, x is the time and X_0 is the time at half height of fluorescence (t_{50}). The k_{app} and t_{lag} can be obtained from $1/\tau$ and $X_0-2\tau$ respectively. Others have obtained alternative empirical parameters [*105*] and a set of master equations to describe the kinetics of fiber formation have been proposed [*103*].

6) Metal ions and Aβ fiber formation in AD

6.1) Cu^{2+} and Aβ, self-association, amorphous aggregation versus fibrillization:

Two studies over a decade ago showed that Zn^{2+} and Cu^{2+} ions caused marked aggregation of the Aβ peptide [*106-108*]. However, these initial studies did not make the distinction between amorphous aggregates, which are thought to be non-toxic to cells, and the formation of amyloid fibers. Further investigations using the fiber specific fluorophore, thioflavin T (ThT), suggested that Cu^{2+} and Zn^{2+} only promote amorphous aggregation of Aβ and actually inhibit fiber formation [*109-113*]. Some of the confusion and misunderstanding in the literature surrounding the area can largely be attributed to using the word 'aggregation' and 'fibrillization' interchangeably. There are a number of studies now published that carefully characterize the promotion and nature of amorphous aggregates generated in the presence of Cu^{2+} [*24, 112-115*]. Studies using primary cell culture and immortal cell lines suggest Cu^{2+} induced amorphous aggregates are non-toxic to cells [*109, 112*].

In contrast to these studies, others have shown that at sub-stoichiometric levels of Cu^{2+} ions Aβ amyloid fiber formation is not inhibited, while supra-stoichiometric levels of Cu^{2+} ions promote amorphous aggregates, however the kinetics of fiber formation were not investigated [*27, 116*]. A key study by Sarell *et al* showed that sub-stoichiometric levels of Cu^{2+} will actually significantly accelerate the kinetics of fiber formation, consistently reducing the lag-time of fiber formation of Aβ(1-40) by more than half [*23*]. Figure 5 shows Aβ fiber growth kinetics, metal-free Aβ preparations typically take more than 70 +/- 2 hours to reach half maximal fluorescence (t_{50}), while the same Aβ preparations with 0.5 or 1 mole equivalent of Cu^{2+} ions cause fibers to form in nearly half the time; 38 +/- 2 hours, at pH 7.4. The lag-time is reduced by Cu^{2+} ions, from 49 to 16 hours. Sub-stoichiometric amounts of Cu^{2+} between 0.2-0.4 mole equivalents display the greatest increase in fiber growth rates. This supports the hypothesis that Cu^{2+} accelerates nucleation as small sub-stoichiometric amounts of Cu^{2+} can nucleate fiber formation. Further addition of Cu^{2+} ions beyond one mole equivalent caused precipitation of Aβ and markedly reduces the amount of fibres generated [*23*], as previously noted [*27, 116*]. Furthermore, at high concentrations of Aβ(1-40) even sub-stoichiometric amounts of Cu^{2+} will inhibit fiber formation [*23, 110, 115*]. It is now clear that at sub-stoichiometric levels of Cu^{2+} to Aβ and more dilute concentrations of Aβ, Cu^{2+} will accelerate the kinetics of fiber formation. While at supra-molecular levels of Cu^{2+} to Aβ or high concentrations of Aβ (40 micro-molar $Aβ_{40}$) [*23, 115*] fiber formation is inhibited. At these conditions, TEM and AFM studies indicate that amorphous aggregation is promoted at the expense of amyloid fiber formation [*24, 112, 113, 115*]. Substoichiometric amounts of Cu^{2+} were also shown to promote Aβ(1-42) fiber formation [*23*], although others have not observed fiber inhibition under quite similar conditions [*112*]. Aβ(1-42) is markedly less soluble than Aβ(1-40) and so the completion favoring amorphous aggregation over fiber formation must be more pronounced. Table 1 highlights some of the studies performed investigating the effect of Cu^{2+} on Aβ(1-40) and Aβ(1-42) fiber formation, highlighting the different effects of Cu^{2+} and Aβ concentrations.

The process of generating amyloid fibers (described as nucleation dependent polymerization) has many parallels with protein crystallization [*1*]. Like amyloid fibril formation crystallization can also be accelerated by a nucleating 'seed'. With protein crystallization, the concentration of the protein, the pH of the solution, and the levels of salts are adjusted to maximize self-association of the protein molecules into ordered crystals. If conditions are created that are too self-associating for the protein then amorphous aggregates are generated rather than crystals,

this is also true in fibrillization; self-association must occur for ordered fibers to form, however, if conditions are too self-associating amorphous aggregates are generated.

The amorphous aggregates of Cu-Aβ are not cytotoxic [*109, 111, 112*], while Aβ preparations generated under sub-stoichiometric amount of Cu^{2+}, that favor amyloid formation, will significantly enhance the cytotoxicity [*23*]. It seems clear that *in vivo*, levels of both Aβ and Cu^{2+} are lower than for the *in-vitro* studies, thus Cu^{2+} is likely to exclusively accelerate fiber formation *in-vivo*.

6.2) Mechanism of Cu^{2+} accelerated amyloid formation:

So what is the mechanism by which Cu^{2+} ions accelerate the rate of fiber formation? A range of possible mechanisms by which metal ions might influence fiber formation kinetics are discussed in general terms in section 2. At micromolar concentrations of Aβ, Cu^{2+} does not form crossed-linked species [*56, 57, 117*]. The Cu^{2+} coordination geometry is identical in the monomer and fiber [*53, 54*]. Most importantly, the affinity of Cu^{2+} for Aβ is identical for monomer and fiber, suggesting the same complex forms without cross-linking [*53*]. Furthermore, without a reductant there are no reactive oxygen species generated and therefore no di-tyrosine cross-linking [*27*], consequently copper bridging to form cross-linked Aβ, as a possible mechanism of accelerated fiber formation, is ruled out. The conformational changes in Aβ upon Cu^{2+} binding are small and outside of the fiber core [*53, 56*], it therefore seems unlikely that the Cu^{2+} coordination triggers the Aβ misfolding directly by a change in main-chain conformation. However, intermolecular self-association is strongly influenced by the net charge of the protein. As Aβ approaches its isoelectric point, a pI of 5.3, and an overall neutral charge, its solubility decreases [*118, 119*]. Furthermore, as the pH drops from 8 to 6 the rate at which fibers form significantly increases, with lag times (t_{lag}) reduced by more than four-fold [*23*]. The pH dependence of the fiber growth rates bears a strong resemblance to the protonation state of the histidine residues (pK_a 6.7) and the N-terminal amino group (pK_a 7.9) within Aβ, consequently, the net charge of Aβ is crucial to its amyloidogenicity.

As with pH, the binding of metal ions will also change the net charge of Aβ. Cu^{2+} (and Zn^{2+}) ions bind to the three histidine residues within Aβ [*10, 22, 46, 53, 54, 56, 57*], at pH 7.4 Aβ's histidine residues are predominately (80 %) deprotonated and neutrally charged, thus coordination of Cu^{2+} (or Zn^{2+}) to Aβ's histidines adds two positive charges. Adding positive charge to Aβ at pH 7.4 makes the Aβ peptide complex more neutral in overall net charge, and

therefore more prone to self-association, with the result that fiber growth times are almost halved. It seems the most probable cause of Cu^{2+} ion accelerated fiber formation kinetics is the reduction in the net charge of Aβ which promotes self-association. It is notable the metal coordination involving amide ligands (as is the case for PrP and αSyn, see Figure 3), will result in deprotonation. Thus the net change in charge upon Cu^{2+} binding may be small, or even cause a loss of positive charge.

6.3) Cu-Aβ and Cell Toxicity

There are a number of studies investigating the effect of Cu^{2+} on the cell-toxicity of Aβ [*23, 109, 112, 120-122*]. At first glance these studies seem to report conflicting observations. In some studies enhanced cytotoxicity is reported for Cu^{2+} plus Aβ [*121*], while others report that Cu^{2+} has a protective effect on Aβ toxicity [*109, 112*]. When the nature of the Aβ preparation is considered (e.g. amorphous aggregates, monomers or fibers) the various studies become more consistent. When supra-stoichiometric levels of Cu^{2+} are used, amorphous aggregates are generated, which are not cytotoxic, but when sub-stoichiometric amounts of Cu^{2+} are used, Aβ fibers are generated and the presence of Cu^{2+} will significantly enhance the cytotoxicity. Sarell *et al* have shown both the protective effect of Cu^{2+} at supra-stoichiometric levels together with enhanced cytotoxicity for sub-stoichiometric levels [*23*], as shown in Figure 6. *In vivo,* Aβ is present at lower levels than for the *in vitro* experiments and sub-stoichiometric levels of Cu^{2+} are the more physiologically relevant case, suggesting a role for Cu^{2+} ions in enhancing Aβ's cytotoxicity.

The mechanism by which Aβ is toxic to cells (irrespective of the presence of Cu^{2+} ions) is hotly debated [*32, 123*]. It is often suggested that Aβ acts at the membrane surface to disrupt its integrity; thinning or forming pores in the plasma membrane to cause membrane leakage and loss of cellular Ca^{2+} homeostasis. One popular hypothesis is that the membrane integrity is compromised by lipid peroxidation from reactive oxygen species (ROS), which is a feature of the pathogenesis of AD and other protein misfolding diseases [*124*]. One key observation shows that hydrogen peroxide mediates Aβ toxicity and the anti-oxidant enzyme catalase protects cells from Aβ toxicity [*40, 121, 122*]. Fenton redox cycling of copper or iron ions are a likely source of extra-cellular H_2O_2 [*125*]. Indeed Cu^{2+} bound to Aβ will readily generate hydroxyl radicals and H_2O_2 in the presence of a physiological reductant such as ascorbate [*28, 121, 126, 127*]. Furthermore, metallothionein-3 (MT3) will competitively bind copper ions in a redox-inactive form, which will suppress Aβ's cytotoxicity [*120*]. An interesting recent study

has highlighted the role of copper bound prion protein in mediating Aβ neuronal toxicity [*42*] and is discussed further in see section 10.3.

The precise reasons for the enhanced cytotoxicity of Aβ in the presence of Cu^{2+} ions are not clear. Aβ could bind Cu^{2+} promoting oligomer and fiber formation, which may result in an increase concentration of Cu^{2+} ions at the neuronal cell surface in a redox active form, where Cu^{2+} would generate toxic hydrogen peroxide and hydroxyl radicals. Aβ oligomers are found clustered at synaptic terminals [*128*] and cause memory loss due to synaptic failure [*31*]. The observation that the anti-oxidant protein catalase and the Cu^{2+} binding metallothionein-3 (MT3) are protective to cell-culture, strongly supports this hypothesis [*40, 120-122*]. Alternatively, the Cu^{2+} ions could alter the morphology of the fiber or increase the level oligomers relative to fibers, promoting an enhanced toxic effect to the cells. The heightened toxicity may be due to a combination of ROS generation and changes in the morphology of fiber/oligomer generated with sub-stoichiometric amounts of Cu^{2+} ions. Cytotoxicity studies by Sarell *et al* suggest that toxicity is not exclusively due to ROS as Aβ toxicity does not increase commensurately with increased loading of Cu^{2+} ions [*23*]. Thus the ability of Cu^{2+} to promote fiber growth (and by inference the interplay between monmer, oligomer and fiber) and the ability of Cu^{2+} to affect fiber morphology appears to be the significant factor in Cu^{2+} / ROS promoted Aβ cell toxicity.

6.4) Zn^{2+} Aβ, fiber inhibition, oligomer promotion

If Cu^{2+} ions accelerate fiber formation at sub-stoichiometric levels, what about other divalent metal ions? Interestingly, Zn^{2+} ions appear to completely inhibit fiber formation even at low Aβ (3 micro-molar) and zinc ion levels [*23*]. Indeed millisecond pulses to Zn^{2+} ions (mimicking pulses of Zn^{2+} that occur at the synapse) can stimulate Aβ aggregation and inhibit fiber formation [*129*]. A recent paper by Chen *et al* also showed little ThT fluorescence in the presence of Zn^{2+}, even at sub-stoichiometric levels of Zn^{2+} [*24*]. Unlike Cu^{2+}, TEM images of Zn^{2+} loaded Aβ showed no fibers were generated. This may be due to the very different complex (at micromolar concentration) between the two metal ions. Cu^{2+} ions form an intra-molecular complex with Aβ [*53, 54, 56, 57*], while at micromolar concentrations of Aβ, it appears Zn^{2+} will form an inter-molecular complex; cross-linking between histidine residues on multiple Aβ molecules [*22, 46, 63, 130*]. It is probable that a cross-linked Zn^{2+}-Aβ species will inhibit amyloids forming by interfering with the regular cross-beta assembly. These *in vitro* experiments were performed at 3 micromolar concentrations of Aβ. It may be that *in vivo,* where Aβ is much less concentrated (0.1 nanomolar), inter-molecular Zn^{2+} complexes may be

less favored and thus the Zn^{2+} might, like Cu^{2+}, form only intra-molecular complex with histidine side chains and so accelerate fiber formation *in vivo* with a similar mechanism to Cu^{2+} ions.

Interestingly, Aβ aggregates generated by the presence of Zn^{2+} showed a positive binding in a dot-blot antibody assay for Aβ oligomers [*24*]. In contrast, the A11 antibody which recognized Aβ oligomers did not bind with metal free Aβ or Aβ loaded with Cu^{2+} or Fe^{3+} [*24*]. TEM images of the Zn^{2+} loaded Aβ showed ring-shape, pore-like oligomers in the pellet of Zn^{2+} promoted Aβ aggregates. The Zn^{2+} induced oligomers resemble previously characterized annular protofibrils [*33*]. It remains to be established if the Zn^{2+} promoted oligomers will affect cell membrane integrity.

6.5) Fe^{3+}-Aβ:

Although Fe^{2+} binds specifically to Aβ [*131*] the affinity of Fe^{3+} for Aβ appears to be very weak [*132, 133*] and thus one might have predicted little evidence for a link between iron homeostasis and AD, however the opposite is the case. Significantly in both fly [*134*], [*26*] and mice [*135*] models of AD, iron regulation has been shown to exacerbate the disease phenotype. For Aβ fibrils generated in the presence of Fe^{3+}, the rate of formation is inhibited [*26*] particularly at above stoichiometric amounts of Fe^{3+} [*24*]. A study by Crowther *et al* has suggested that Fe^{3+} influences the morphology of Aβ fibers with shorter more curved fibers generated, and it is suggested that it may be this which influences Aβ toxicity in the fly [*26*].

6.6) Ca^{2+} Aβ

Ca^{2+} is found at very high concentrations extra-cellularly, relative to transition metal ions, typically at 2 millimolar in the extra-cellular space. Interestingly *in vitro* Ca^{2+} can accelerate Aβ fiber formation [*136*]. Ahmad *et al* have shown that at physiological levels of Ca^{2+} (2 millimolar), the kinetics of Aβ(1-42) amyloid fiber formation is greatly accelerated, doubling the lag-time [*137*]. Surprisingly, no such effect was observed for Ca^{2+} addition to Aβ(1-40) solutions [*137*]. Ca^{2+} is a hard metal ion and coordinates via oxygen ligands. There are six Asp/Glu residues, largely in the N-terminal half of Aβ, with which Ca^{2+} can coordinate. It is notable that Ca^{2+} (like Cu^{2+}) binding will add positive charge to Aβ at pH 7.4, making it more neutrally charged and, therefore, more prone to self-association. Whether Ca^{2+} homeostasis has an influence on the pathology of AD is not established. Although Ca^{2+} is an abundant bulk metal ion, it is not clear if AD pathology will be strongly influenced by fluctuations in Ca^{2+}

levels outside the cell. However, the high extra cellular levels of Ca^{2+} may influence the critical concentration for fibril formation and could explain why sub-nanomolar levels of extracellular Aβ may be sufficient to cause fibers to form *in vivo.*

6.7) Al^{3+} Aβ:

Fibril growth kinetics, using ThT, indicate little effect on the rate of fiber formation for Ab:Al^{3+} ratios of 1:1 or 1:4. At higher levels of Al^{3+}, fiber formation is strongly inhibited [*24*]. The influence of Al^{3+} on AD aetiology is reviewed in detail elsewhere in the special issue.

7) Metal ions and amyloid formation in transmissible spongiform encotholthapies (TSE)

Although the coordination geometry and affinity of Cu^{2+} binding to PrP^{C} has been studied extensively [*16, 50, 51, 85, 138, 139*], there are surprisingly few studies of Cu^{2+} binding to prion protein fibers or studies on the influence of metal ions on the kinetics of fiber formation. It has been shown that Cu^{2+} promotes aggregation of PrP, lowers PrP solubility and promotes self-association [*140-142*]. In addition, Cu^{2+} ions can convert the PrP^{C} into a protease-resistant species [*140, 143-145*], which is a feature of PrP^{Sc}. Furthermore, Cu^{2+} binding encodes features that correlate with various strains of prion disease [*20*]. There is a report using dynamic light scattering that indicates at millimolar levels, Cu^{2+} promotes oligomers of PrP, while the presence of amyloids were not well characterized [*140*]. Furthermore, Cu^{2+} has been shown to promote the formation of soluble oligomers at pH 5, these Cu-PrP oligomer will reduce cell viability in a SKNSH cell line [*146*].

A careful study by Baskakov *et al* showed that at 1 micromolar Cu^{2+} levels, 1:1 ratio with PrP^{C}, there appears to be little effect on prion protein fiber kinetics. At 10 mole equivalents, Cu^{2+} fiber formation was inhibited and at 100 micromolar Cu^{2+} (1 micromolar PrP), no fiber formation was observed. [*147*]. This behavior has some parallels with Cu^{2+} binding to Aβ as surpra-stoichiometric ratios of Cu^{2+} cause self-association of Aβ into amorphous aggregates and so inhibit fibrillization [*23*]. As with all *in vitro* studies there remains a question as to how well the *in vitro,* observations reflect the *in vivo* situation. Cu^{2+} induced self-association of PrP in the form of amorphous aggregates hints at the possibility that *in vitro* where PrP^{C} is at lower concentrations anchored to the plasma membrane surface, fibrillization might occur rather than aggregation, as is the case for Aβ [*23*]. Interestingly, the same Baskakov *et al* study showed that once amyloid fibers are formed, the presence of Cu^{2+} can affect the morphology of the fibers. Cu^{2+} enhances PK-resistance of preformed fibrils and initiates aggregation of preformed PrP fibrils into larger plaque like clumps [*147*]. Baskakov *et al* also showed that Zn^{2+} at 1:1

ratios has little effect on fibrillization, but at higher levels inhibited fiber formation, but to a lesser extent than Cu^{2+} ions. Mn^{2+} had little effect on fibrillization rates even at 100 mole equivalents [*147*].

8) Metal ions and amyloid formation of αSyn in Parkinson's disease (PD)

Metals have also been proposed as triggers for other misfolding and assembly diseases, including Parkinson's disease (PD). The acceleration of the kinetics of fiber formation of αSyn was described over ten years ago [*104, 148, 149*]. However, these early studies used high levels of metal ions, higher than those normally found physiologically. Using 0.5-5 mM concentrations, a number of transition metal ions increased the rate at which αSyn formed amyloid fibers. Significantly, subsequent studies by Fernandez *et al*, using much lower concentrations of Cu^{2+} ions also accelerated fiber formation. [*89*] They showed that a 1:1 binding of Cu^{2+} with 100 micromolar of αSyn will significantly reduce the lag-time of fibrillization. A follow up study investigating the effect of other divalent transition metal ions; Mn^{2+}, Fe^{3+}, Co^{2+} and Ni^{2+} showed little or no effect on the kinetics of αSyn fibril formation using 1:1 metal:αSyn ratios [*99*]. These metal ions have very low affinities for αSyn in the millimolar range, and as a consequence, at these more physiologically relevant levels (100 micromolar αSyn) there are few of these metal ions bound to αSyn. Furthermore, Cu^{2+} will exacerbate the cytotoxicity of αSyn as assessed by additions of Cu-αSyn to cell culture [*150*].

9) Metal ions and amyloid formation in other protein misfolding diseases

Metals have also been proposed as triggers for other misfolding and assembly diseases such as dialysis-related amyloidosis [*21*]. Here Cu^{2+} binds to β2-microglobulin (β2m) and causes a key *cis-trans* isomerization of a proline at position 32. Another protein perturbed by metal ions is the human islet amyloid peptide (hIAPP); a highly amyloidogenic peptide found in the islet cells of patients with type-II diabetes. hIAPP is toxic to β-cells and is linked to a loss of insulin secretion. Although highly amyloidogenic, hIAPP is safely stored in the secretory granules at high concentrations. Interestingly, unusually high millimolar levels of Zn^{2+} are found in pancreatic β-cells. It has been shown that these high levels of Zn^{2+} will inhibit hIAPP fiber formation. This raises the possibility that zinc has a protective role in hIAPP amyloid formation associated with type-II diabetes [*151, 152*]. Cu^{2+} will also inhibit hIAPP amyloid formation [*153*]. Aberrant metal binding is also implicated in the misfolding and accumulation of Cu-Zn-superoxide dismutase (SOD) in amyotrophic lateral sclerosis (ALS) [*154*]. While

Huntington's Disease, a poly-glutamate protein misfolding disease, has also been linked with copper promoted aggregation [*155*].

10) Metal ion homeostasis at the synapse and metal ion affinities for Aβ and PrP

From the previous sections it clear there is now compelling data to show that metal ions influence, and can accelerate fiber formation and enhance cytotoxicity. However, a role for metal ions in protein misfolding diseases remains controversial, as it is often believed that the affinity of Aβ and PrP for metal ions is not sufficiently high to be physiologically relevant. This section highlights some pertinent features of metal ions at the synapse and protein affinities.

10.1) Cu^{2+} and Zn^{2+} at the synapse:

Aβ and PrP are found concentrated at the synaptic cleft, as shown in Figure 7. PrP^{C} is found bound to the extra-cellular membrane, while Aβ is released at the synapse [*128, 156, 157*]. The concentrations of metal ions at the synapse are therefore of particular interest as it is now established that Zn^{2+} and Cu^{2+} are released at the glutamatergic synapse in the cortex and hippocampus. There are now a number of excellent reviews describing what is known about Zn^{2+} and Cu^{2+} at the synapse [*8, 158-160*]. This synapse is the site of long-term potentiation, which is responsible for memory formation and it is here that Aβ amyloid deposits are first observed in AD patients. Zn^{2+} ions are released, possibly with glutamate, during neurotransmission [*161*], fluxes of Zn^{2+} released into the extracellular space may be as free ionic form or as exchangeable Zn^{2+} at 10-30 micromolar levels [*162*]. It is believed Zn^{2+} will reach even higher levels during brief synaptic release events, perhaps 100-300 micromolar [*158, 163, 164*]. Similarly there is a release of exchangeable copper post-synaptically following activation of the NMDA receptor [*165-167*], Figure 7. It is believed Cu^{2+} reaches levels of 15 micromolar [*168*], some have reported even higher fluxes of Cu^{2+} from 20-250 micromolar, during neuronal depolarization [*17, 169*].

10.2) Aβ and PrP affinity for metal ions:

A role for metal ions in protein misfolding is often disputed on the basis of insufficient metal – protein affinity. Affinity measurements are a potentially difficult area and are often plagued with conflicting observations. An excellent review that highlights some of the common potential pitfalls in metal affinity measurements and calculations see [*170*]. For example, often the effect of competing buffers, multiple binding modes of competing ligands, the solubility of the metal ion and competing ligand, or its oxidation state as well as the pH dependence on the

affinity are sometimes incorrectly understood or simply not considered. There have been numerous metal affinities reported for Aβ, PrP^C and αSyn over the past decayed but now perhaps a consensus is emerging. Table 2 highlights some key measurements of conditional dissociation constants at pH 7.4 for copper binding to Aβ , PrP^C and αSyn. Affinities shown in Table 2 are by no means a comprehensive list, it's purpose is to highlight some of the key recent studies. For a more complete review of this area and a discussion of conditional binding constant that take into account the competitive effects of buffers , see Faller *et al* [*46*].

The affinity of Cu^{2+} for Aβ has been calculated using 3 different competing ligands; very similar K_ds are observed for monomeric and fibrilar Aβ for all 3 competing ligands. Setting the conditional dissociation constant, pH 7.4, at 54 +/-5 picomolar (54×10^{-12} M) [*53*]. The use of three different competitors for Cu^{2+} that all indicate almost identical affinities for the Cu^{2+}-Aβ complex indicates that a ternary complex, which might complicate the determination of a Cu^{2+} affinity, does not from. Hatcher *et al* [*171*] and Tougo *et al* [*172*] have reported Cu^{2+} affinities a single order of magnitude weaker.

Extra-cellular monomeric Aβ levels are thought to be 0.1-1 nanomolar [*173, 174*], while Aβ levels are higher in plaques and at the synapse. Extra-cellular Cu^{2+} levels in the brain interstitial fluid are typically 100 nM. A picomolar affinity for Cu^{2+} allows Aβ to compete for these ions with other extracellular Cu^{2+} chelators, especially at the synapse during neuronal depolarization where fluxes of Cu^{2+} are reported to be 20-250 micromolar and Aβ is localized [*169*].

Zn^{2+} affinities for Aβ are relatively weak, with 1-20 micromolar dissociation constants reported [*46, 175*] . However, it is known that exchangeable or free Zn^{2+} ions can reach levels as high as 30-300 micromolar at the glutamatergic synapse [*158, 163, 164*]. Thus Zn-Aβ interactions may also be physiologically relevant.

PrP^C has as many as six binding sites with 30-100 nanomolar affinities [*85*]. Others have reported Kds to within one or two orders of magnitude [*72, 73, 176*]. PrP^C is situated at the plasma membrane of the synapse, with 6 potential Cu^{2+} binding sites on the flexible N-terminal domain, it must, therefore, make it a good buffer for micro-molar fluxes of Cu^{2+} at the synapse. Cu^{2+} is bound to PrP^C *in vivo* [*17*] and furthermore Cu^{2+} and Zn^{2+} will trigger endocytosis of

PrP^{C}. Rapid turnover of PrP and increased oxidative stress associated with PrP knockouts suggest that PrP might have a protective anti-oxidant role [*29, 177, 178*].

To make a prediction as to the likely levels of metal binding to PrP and Aβ *in-vivo* we also need to consider the levels of other metallo-proteins at the synaptic cleft. Metallothionein3, (MT3) found within neurons may be to buffer synaptic fluxes of Cu^{2+} and Zn^{2+} as it is released in the cleft by near-by astrocytes [*179*]. MT3 binds Cu^{2+} in a redox inactive state and can protect against the toxic effects of Cu^{2+} and Zn^{2+} [*120, 158*]. Interestingly, MT3 levels are suppressed in AD patients [*180*], although this has been disputed [*181*]. In this situation, the buffering of these metal ions may become swamped and therefore available for binding Aβ and PrP. Humans serum albumin highly concentrated in blood plasma, at 640 micromolar, and binds extra-cellular Cu^{2+} with a 1 picomolar affinity. However, the concentration of albumin is considerably lower in the CSF, (3 micromolar) and so may be swamped by spikes of Cu^{2+} at the synapse during neuronal depolarization, Figure 7.

10.3) Aβ toxicity mediated by copper bound PrP^{C} at the synapse

Interestingly, both PrP^{C} and Aβ are concentrated at the synapse and there is now strong evidence to link Aβ neurotoxicity with the presence of PrP^{C} [*182, 183*]. Aβ toxicity in mice models of AD requires the presence of PrP^{C} [*182*]. Indeed PrP knockout mice can develop Aβ plaques but not neurotoxicity [*184*]. Interestingly PrP^{C} selectively binds to Aβ oligomers [*185, 186*]. A molecular mechanism for the PrP^{C} dependent Aβ toxicity has recently been proposed which indicates Aβ disrupts copper homeostasis at the synapse which is required for normal PrP^{C} dependent inhibition of excessive N-methyl-D-aspartate receptors (NMDAR) activity [*42*]. NMDA receptors mediate critical CNS functions, a physiological role for PrP^{C}, has been proposed that limits excessive NMDAR activity that might otherwise promote neuronal damage [*187*]. Significantly, PrP^{C} only affects the NMDA receptor in a copper-loaded state [*42*]. There is evidence to suggest that the oligomeric form of Aβ released at the synapse, with a picomolar affinity for Cu^{2+}, may disrupt Cu^{2+} binding to PrP^{C} and so, in part at least, mediate neuronal and synaptic injury [*42*].

11) *In vivo* evidence for a link between metal ions and protein misfolding diseases:

From the previous section it is clear that the affinities of Aβ and PrP for Cu^{2+} and Zn^{2+} ions are sufficiently tight for them to be physiological relevant, particularly when fluxes of these ions are released at the synapse during neuronal depolarization. In this section evidence, from *in*

vivo studies, in support of a role for metal ions in a number of protein misfolding diseases are highlighted.

11.1) Alzheimer's disease (*AD):*

Copper: In vivo studies using a *Drosophila* model of AD have shown that impaired copper homeostasis enhances the toxic effects of Aβ [*188*], while a rabbit model of Alzheimer's disease showed rabbits fed copper in a high cholesterol diet develop amyloid plaques and learning deficits [*189*]. In contrast, transgenic mice have shown a reduced AD pathology with increased intra-cellular copper levels [*190-192*]. Interestingly Cu^{+} will also bind to Aβ and may therefore influence fiber formation [*193*]. Understanding the interplay between intra and extra cellular copper and its effect on Aβ will be essential for a more complete picture of copper related AD pathology [*194*]. It appears that AD may be characterized by an increase in labile extra-cellular pool of Cu^{2+} ions [*195*].

Zinc: Zn^{2+} is concentrated in synaptic vesicles with the assistance of a specific zinc transporter, Zn-T3. Significantly, a mouse model of AD with Zn-T3 knocked out do not develop amyloid plaques in the brain [*196*]. Furthermore, MT3 is released by astrocytes at the synaptic cleft and have a role in buffering Cu^{2+} and Zn^{2+}. Interestingly, MT3 (GIF) levels are reduced by an order of magnitude in AD patients [*180*] although this is contested [*181*].

Iron: Significantly, in both fly [*26, 134*] and mice [*135*] models of AD, iron regulation has been shown to exacerbate the disease phenotype; reducing fly longevity and increasing rough eye. In particular, ferritin, the iron storage protein, will influence the disease phenotype [*26, 134*].

11.2) TSE:

The relationship between metal ions and prion disease have been reviewed [*50, 51, 138, 139*]. A feature of prion disease are metal imbalances [*19*]. Copper shows an increase in toxicity for PrP knockout mice and exacerbates disease in a mouse model of familial CJD [*197*]. Increased copper in the diet of mice raises PrP^{C} levels; although counter intuitively survival times after scrapie infection are reduce with mice fed a low copper diet [*18*]. Furthermore, when isolated from diseased brain, PrP^{Sc} has been found to be occupied with Cu^{2+} ions [*20*]. Different strains of prion disease may be generated depending on the presence, or absence, of Cu^{2+} ions [*20*]. PrP knockouts show altered metal ion homeostasis in the mice brain [*198*]. Protease resistance in PrP is induced upon Cu^{2+} binding [*144, 145*]. Younan *et al* have recently shown Cu^{2+} ions

can destabilize the fold of PrP^{C}. The free energy of folding is lowered by 2 kJ/mol and therefore may make the transition to misfolded PrP^{Sc} more thermodynamically favorable [*25*]. A form of familial prion disease is linked to an increased ability to bind Cu^{2+} ions with additional octarepeats present, while Cu^{2+} binding to the amyloidgenic fragment of PrP is linked to increased neurotoxicity [*199-201*]. In addition, oxidative modifications of PrP, generated by copper catalyzed Fenton reaction has been linked to prion disease [*29, 30, 202*]. Significantly, copper ion chelation therapy will delay onset of scrapie in mice [*203*].

11.3) Parkinson's Disease (PD) :

An increased risk of developing PD has been linked to industrial exposure to heavy metal ions [*204*]. Elevated levels of Cu^{2+} have been reported in the cerebrospinal fluid of PD patients [*14*], in addition iron deposits have been found in the lewy bodies [*15*]. Further details are discussed elsewhere in this special issue.

12) Is there a shared metal associated mechanism of amyloidoses?

It is believed that all proteins under the right conditions will form amyloid structures with a shared generic cross-beta structural motif [*1*]. Perhaps the mechanism by which metal ions accelerate fiber formation is also shared. Sarell *et al* has proposed that metal accelerated fiber formation for Aβ might be driven by electrostatics [*23*]. At pH 7.4 metal ion coordination via imidazole side chains will cause the net charge of the protein to be more positive. However this is not always the case, if coordination is dominated by amides, as in the case of for some of the modes of coordination of Cu^{2+} to the prion protein (Figure 3) then Cu^{2+} binding will actually reduce the net charge, due to the loss of up to three protons upon forming the complex. Thus the change in net charge of the protein upon metal coordination is dependent on the type complex formed.

Theoretical pI's for a number of amyloidogenic proteins are given in Table 3. Aβ and αSyn, have acidic pI's, 5.3 and 4.6 respectively. Using our understanding of the coordination geometry it appears Cu^{2+} at pH 7.4 will make Aβ more neutrally charged. However the first equivalent of Cu^{2+} binding at the N-terminus of α-Syn will have little effect on the overall charge due to the displacement of two protons, one from the amino group and one from an amide (Figure 3). The prion protein possesses a basic pI, 9.8 so a neutral pH PrP is already positively charged, however Cu^{2+} binding dominated by amide proton displacement will make PrP more neutrally charged binding centred at His^{111} or His^{96} of PrP. Unlike Aβ and αSyn, Cu^{2+} binding to PrP have been reported to inhibit fiber formation [*147*].

The acceleration of fiber formation upon metal bind may be a combination of a number of effects, and a change in net charge is just one of these. It does not appear that all metal associated amyloidosis are related to a change in net charge and increased self-association. Cu^{2+} binding to β2m appears to be closely related to a structural rearrangement induced by Cu^{2+} binding rather than a change in net charge [*21*]. While in SOD, it is the destabilization of the native fold of the protein due to a point mutation and a loss of a zinc ligand that destabilizes the native protein fold [*154*].

13) Concluding remarks

Metal ions are capable of affecting amorphous aggregation, oligomerization and fibrillization of a number of amyloidogenic proteins associated with protein misfolding [*21, 89, 147, 151*]. Furthermore, metal ion homeostasis, particularly at the synapse, where Zn^{2+} and Cu^{2+} are released as a labile pool is now being recognized as a potential risk-factor in these multi-factorial diseases. In particular, for Alzheimer's disease (AD), it is now established that Aβ has a tight, physiologically relevant, picomolar affinity for Cu^{2+} [*53*]. Furthermore, Cu^{2+} binding significantly accelerates the rate of fiber formation and enhances cytotoxicity in cell culture [*23*]. These observations are supported by *in vivo* studies in animal models of AD that implicate Cu^{2+} impaired homeostasis in promotion of the disease [*188, 189*]. A recent study suggests the toxicity of Aβ is mediated by both copper ions and the prion protein [*42*]. This is a particularly exciting new development linking copper homeostasis at the synapse with two amyloidogenic proteins, and is set to generate a major paradigm shift in our understanding of Alzheimer's disease.

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Figure Captions

Figure 1: **Potential mechanisms by which metal ions can perturb protein fiber formation**: i) Metal induced conformational change. ii) Metal induced cross-linking. iii) Metal induced neutral net charge iv) Metal induced change in fiber morphology. v) Metal induced change in protein stability.

Figure 2: Fibers *versus* amorphous aggregates. Fibers are in equilibrium with, oligomers, monomers and amorphous aggregates and there relative proportions are interdependent.

Figure 3: Cu^{2+} coordination to Aβ, PrP and αSyn. These are models adapted from [*53, 55*] for Aβ [*16*] for the prion protein modes of coordination (mouse sequence numbering) and [*94*] for α-synuclein.

Figure 4: Fiber growth kinetics. Nucleation–polymerization reaction causes a sigmoidal growth curve with a lag-phase and elongation. Fiber formation is initially very slow until nucleating seeds are generated; this initiate's rapid fiber generation until equilibrium with low amounts of protein monomer is reached. Agitation will cause fragmentation and additional secondary nucleation and a reduced lag-phase.

Figure 5: Cu^{2+} accelerates fiber growth. (a) Average of 9 growth curves recorded on two separate occasions, apo in red, 1 mole equivalent Cu^{2+} in blue. The fluorescence ThT signal is normalized at maximal intensity. Aβ(1-40) 5 micromolar, HEPES buffer 50 mM, 160 mM NaCl at 30 ^{o}C. (b) Time to reach half-maximal fluorescence (t_{50}) in two experiments with 0 (red), 1 (mid-blue) or 0.5 (dark blue) mole equivalents of Cu^{2+} ions. The presence of Cu^{2+} typically halves the time taken to form fibers. Error bars are for standard error (s.e.m.) from nine traces. P= 0.001 indicated by ***. Adapted from [*23*].

Figure 6: Cell viability, Cu^{2+}-Aβ is more cytotoxic than Aβ. Aβ(1-42) as preformed fibrils, 3 micromolar, with and without the presence of 1.5 micromolar Cu^{2+} were added to PC12 cells. Cu^{2+} was added to the cells alone to test Cu^{2+} toxicity. Blank is buffer only. All preparations were incubated with the cells for 24 hrs then 10% (v/v) Alamar Blue was added. The data shown here is after total incubation for 70 hrs. Error bars are standard error (s.e.m), n=3. P= 0.001 indicated by ***. Adapted from [*23*].

Figure 7: Zn^{2+} and Cu^{2+} at the synapse. Exchangeable Zn^{2+} is released with glutamate from vesicles at gutamergic synapse after neuronal excitation, 30-100 micromolar spikes of Zn^{2+} may be reached. Cu^{2+} is released postsynaptically after NMDA receptor activation, 15 micromolar or more fluxes of Cu^{2+} are reported. Aβ is cleaved from APP in to the lumin of vesicles as it travels down the axon, Aβ is then released at the presynapse . PrP is anchored to the plasma membrane concentrated at the presynapse. ZnT-3 and the menkes protein, ATP7a, are key protein responsible for trafficking Zn^{2+} and Cu^{+} respectively into vesicles. Metallothionein3 (MT3), released from astrocytes may serve as a buffer for the fluxes of Zn^{2+} and Cu^{2+} but may be depleted in Alzheimer's disease.

Table 1: The effect of Cu^{2+} on Aβ fiber formation.

Stoichiometry [Cu^{2+}]/ [Aβ]	**Peptide**	**Concentration** (micromolar)	**Technique**	**Observation**	**Ref**
0.2	Aβ(1–40)	5	ThT	Accelerate fiber kinetics	[*23*]
0.5	Aβ(1–40)	5	ThT	Accelerate fiber kinetics	[*23*]
1	Aβ(1–40)	5	ThT	Accelerate fiber kinetics	[*23*]
0.5	Aβ(1–42)	3	ThT	Promotes fibers	[*23*]
2	Aβ(1–40)	5	ThT	No fibers	[*23*]
0.5	Aβ(1–40)	50	ThT	No fibers	[*23*]
2	Aβ(1–40)	10	ThT	No fibers	[*109*]
2	Aβ(1–42)	10	ThT	Reduced fibers	[*109*]
0.5	Aβ(1–42)	5	ThT	Reduced fibers	[*112*]
1.0	Aβ(1–42)	5	ThT	No fibers	[*112*]
1	Aβ(1–40)	25	ThT	Reduced fibers	[*24*]
0.2	Aβ(1–40)	25	TEM	Fibers observed	[*24*]
0.4	Aβ(1–40)	50	ThT	No fibers	[*110*]
0.2	Aβ(1–40)	50	ThT	Reduced fibers	[*110*]
1	Aβ(1–42)	40	ThT	Reduced fibers	[*115*]
<1	Aβ(1–42)	--	TEM	Fibers observed	[*27*]
>1	Aβ(1–42)	--	TEM	No fibers	[*27*]
1	Aβ(1–40)	--	TEM	Fibers observed	[*116*]
2	Aβ(1–40)	--	TEM	No fibers	[*116*]
0.5	Aβ(1–42)	--	AFM	No fibers	[*113*]
0.7	Aβ(1–40)	2.5	Centrifugation	Increased precipitation	[*107*]
1	Aβ(1–40)	20	Absorption	Increase turbidity	[*108*]

The data is largely consistent with supra-stoichiometric amounts of Cu^{2+} or high concentration of Aβ generating amorphous aggregates. While at physiologically relevant lower concentrations and substoichiometric Cu^{2+} amyloid fibers are observer and their rate of formation is accelerated.

Table 2: Copper^{2+} affinities for synaptic amyloidogenic proteins.

Protein	**Conditional $K_{d,}$ pH 7.4**	**Reference**
Amyloid beta peptide (Aβ)	54 picomolar	[*53*]
	400 picomolar	[*171*]
	35 nanomolar (* 625 picomolar)	[*172*]
Prion protein (PrP^C)**	30 nanomolar	[*85*]
	3 nanomolar	[*72*]
	0.1 nanomolar	[*73*]
Alpha-synuclein (αSyn)	0.4 nanomolar	[*97*]
	0.2 nanomolar	[*98*]
	0.1 nanomolar	[*94*]

Data presented are for conditional dissociation constant at pH 7.4. *Correction made for the buffer used. **PrP^C binds up to six Cu^{2+} ions, a weaker affinity of 100 nanomolar is reported for Cu^{2+} binding to single octarepeats.

Table 3: pI's for amyloidogenic proteins and the effect of Cu^{2+} on fiber growth rates.

Protein	**pI**	**Effect of Cu^{2+} on Fiber growth kinetics**	**Reference**
Amyloid-beta peptide (Aβ)	5.3	Accelerate	[*23*]
Alpha-synuclein (αSyn)	4.4	Accelerate	[*89*]
β2-microglobulin (β2m)	6.5	Accelerate	[*21*]
Human Prion protein (PrP)	9.8	Inhibit	[*147*]
islet amyloid poly peptide (IAPP)	8.9	Inhibit	[*153*]

For protein with acid pI's amyloid fiber formation is accelerated while for basic proteins it is inhibited.

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