The metallobiology of Alzheimer's disease

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The cause of Alzheimer's disease (AD) is closely related to the aggregation of a normal protein, β-amyloid (Aβ), within the neocortex. Recently, evidence has been gathered to suggest that Aβ precipitation and toxicity in AD are caused by abnormal interactions with neocortical metal ions, especially Zn, Cu and Fe. However, Aβ might also participate in normal metal-ion homeostasis. An inevitable, age-dependent rise in brain Cu and Fe might hypermetallate the Aβ peptide, causing the catalysis of H_2O_2 production that mediates the toxicity and auto-oxidation of Aβ. The greater incidence of AD in females could be due to greater constitutive activity of the synaptic Zn transporter ZnT3, and attenuated binding of metal ions to the rodent homologue of Aβ might explain why these animals are spared Alzheimer's pathology. Compounds that interdict metal-ion binding to Aβ dissolve brain deposits *in vitro* and one such compound, clioquinol, inhibits Aβ deposition in the Tg2576 mouse model for AD and could be useful clinically. These insights could also apply to other degenerative disorders in which metal-ion–protein interactions have been implicated.

Current drug therapies for Alzheimer's disease (AD) do not inhibit the unremitting neuropathology of the disease. Modern approaches propose to target the underlying disease process that genetic evidence links to the accumulation of β-amyloid (Aβ) in the neocortex, the pathological hallmark of the disease (reviewed in Ref. [1]). However, the factors that drive Aβ accumulation and how this relates to dementia have remained contentious.

The 'amyloid cascade' theory, which has dominated AD research for the past 15 years, proposes that Aβ is protein junk that spontaneously self-aggregates into amyloid fibrils that are somehow neurotoxic and cause dementia. The longer forms of Aβ (mainly $Aβ_{1-42}$) are regarded as particularly pathogenic because they are overproduced as a result of familial gene mutations, and are more apparently self-aggregating than are shorter forms (e.g. $Aβ_{1-40}$) in *in vitro* studies (reviewed in Refs [1,2]). On the basis of the amyloid cascade model, the main therapeutic strategies have aimed either to prevent the production of Aβ [with inhibitors of β- and γ-secretases that generate Aβ from its ubiquitous precursor, amyloid precursor protein (APP)], or to remove all forms of Aβ (e.g. the Aβ 'vaccine' [3]). However, Aβ is ordinarily produced in the brain, and there is no proof that Aβ overproduction underlies sporadic AD (reviewed in Refs [1,2]).

The self-aggregation hypothesis leaves important questions about the biology of AD unanswered. Why does Aβ, which is ubiquitous, precipitate only in the neocortex? Why are age and gender (elderly and female) major risk factors for AD and cerebral amyloid deposition in both humans and APP transgenic mice [4]? Why do rats and mice, unlike other mammalian species, not develop Aβ neuropathology with advancing age [5]? The rapidly growing knowledge of the metallochemical properties of Aβ, combined with advances in research of the neurobiology of metal-ion metabolism in the brain, has provided a model for AD that presents cohesive explanations for these mysteries.

Metallochemistry mediates the aggregation and toxicity of Aβ in AD

Aβ has been described in three biochemical fractions in the brain: membrane associated, aggregated and soluble. In healthy individuals, most of the Aβ is membrane associated, but in individuals with AD the aggregated (diffuse and plaque amyloid) and soluble fractions increase markedly [6,7]. Zn^{2+} appears to be the major neurochemical factor responsible for aggregating Aβ. Originally, *in vitro* studies found that Zn^{2+}, at low micromolar concentrations, rapidly precipitated soluble Aβ into protease-resistant amyloid aggregates *in vitro* [8,9]. Intermolecular His(Nτ)-Zn^{2+}–His(Nτ) bridges are formed in this reaction [10]. Although Zn^{2+} is the only physiologically available metal ion to precipitate Aβ at pH 7.4 [9,11], Cu^{2+} (and Fe^{3+} to a lesser extent) induces limited Aβ aggregation, which is exaggerated by slightly acidic conditions [11]. Zn, Cu and Fe*, unlike heavy metal ions or aluminium, are constitutively found at high levels in the neocortical regions most prone to AD pathology (Fig. 1a), where they play important roles in normal physiology. Cu^{2+} [12,13] and Zn^{2+} [14,15] are also released during neurotransmission. In accordance with our predictions, Zn (Fig. 1b), Cu and Fe are found at markedly high levels in cerebral Aβ deposits [16,17], and

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*Chemical symbols for zinc (Zn), copper (Cu) and iron (Fe) are used to refer to the metals in their ionic state, for which Cu and Fe can be in more than one valence state (Cu^{+} or Cu^{2+} and Fe^{2+} or Fe^{3+}, respectively).

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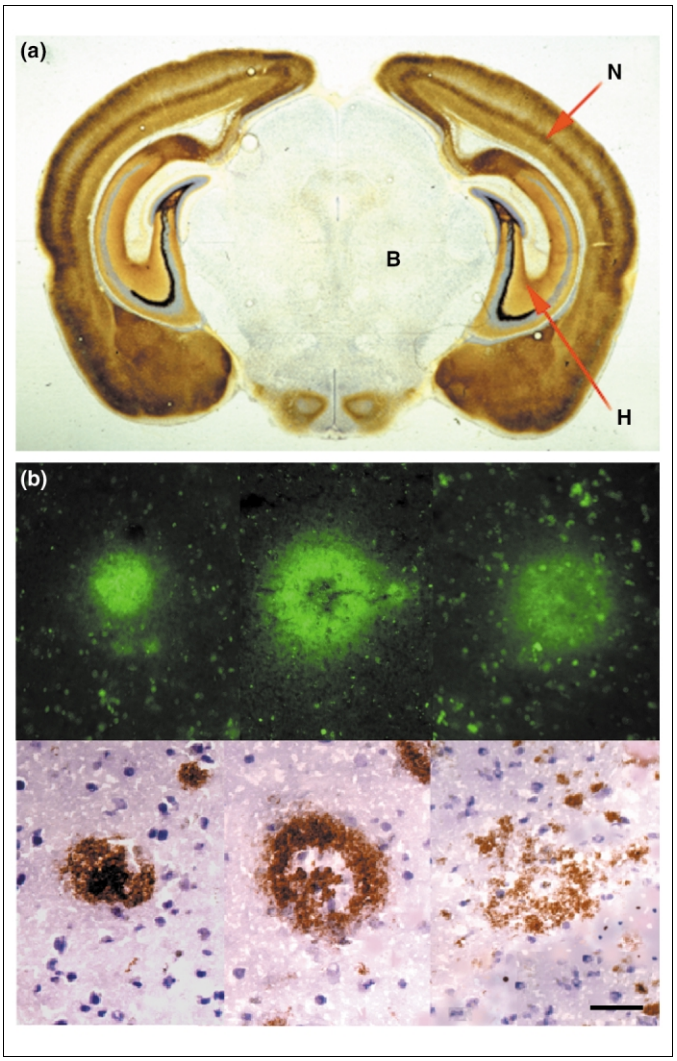

Fig. 1. Brain Zn^{2+} localization in normal neocortex and in an Alzheimer's disease (AD) amyloid plaque. (a) Timm's stain for pools of dissociable ionic Zn^{2+} or low-affinity-bound Zn^{2+} in adult rat brain (coronal section). The stain detects mainly Zn^{2+} within glutamatergic synaptic vesicles in the neocortex, representing ~30% of total brain Zn (much of the remainder is tightly bound to proteins and not detected by the stain). This Zn^{2+} is transported into the vesicles by ZnT3. Genetic ablation of ZnT3 inhibits β-amyloid deposition in Tg2576 mice [24]. Abbreviations: B, basal ganglia; H, hippocampus; N, neocortex. (b) Zn staining of amyloid plaques in AD. Three typical plaques (left to right) are stained with TSQ fluorescent stain for Zn [17] (top panel) and anti-Aβ antibody (4G8, bottom panel). Scale bar, 100 μm. Photomicrograph courtesy of Christopher Frederickson (NeuroBioTex, Galveston, TX, USA).

Cu and Zn co-purify with Aβ from brain tissue of individuals with AD [18]. Importantly, rat and mouse Aβ, which possesses three amino acid substitutions that abolish the crucial bridging histidine 13 [19–21], are not precipitated by Zn^{2+} or Cu^{2+} at physiological concentrations [9,11]. This explains why mice and rats do not deposit cerebral Aβ amyloid [5], even when mouse $Aβ_{x-42}$ is overproduced in presenilin mutant mice [22].

Zn release during synaptic transmission induces cerebral β-amyloid deposition in the Tg2576 transgenic mouse model for AD. In a study that crossed these mice with *ZnT3*-knockout mice (which lack synaptic Zn [23]), cerebral Aβ deposition was nearly abolished in the Tg2576/*ZnT3* $^{-/-}$ progeny [24]. ZnT3 loads Zn^{2+} into synaptic vesicles [25] within glutamatergic corticofugal fibers (Fig. 1a), which represent ~30% of brain Zn [26]. During neurotransmission, extracellular Zn concentrations reach ≈300 μM [26] in the neocortex. This Zn^{2+} is in a low-affinity bound or exchangeable chemical form, and possibly co-released with glutamate. Zn^{2+} reuptake after synaptic release is rapid and energy dependent, maintaining minimal basal levels in the interstitial spaces that are estimated to be <500 nM (reviewed in Refs [26,27]). ZnT3 is situated in the vesicular membrane, but the mechanism of its participation in transport of Zn^{2+} into the synaptic vesicle remains to be elucidated. This efficient, energy-dependent Zn^{2+} flux would be a vulnerable site for energy depletion, which could cause pooling of extracellular Zn and the initiation of Aβ deposition (Figs 2,3). Zn release during neurotransmission might also explain the gender effect of AD because female mice exhibited age-dependent hyperactivity of the ZnT3 transporter associated with increased amyloid deposition, which was abolished in Tg2576/*ZnT3* $^{-/-}$ mice [24].

Less is currently known about extracellular Cu in the neocortex, but *in vitro* evidence suggests that cortical tissue depolarization releases Cu to achieve micromolar concentrations, although it is not yet clear if this Cu is in a chemically exchangeable form [13]. However, biochemical studies strongly imply a crucial role for Cu^{2+} and Fe^{3+} interaction with Aβ species in AD. The affinity of variant Aβ species (e.g. $Aβ_{1-40}$ and $Aβ_{1-42}$) for Zn^{2+} is equal (A.I. Bush, unpublished), but the affinity for Cu^{2+} and Fe^{3+} differentiates variant Aβ species in manner that echoes their participation in AD pathology [$Aβ_{1-42(human)} > Aβ_{1-40(human)} > Aβ_{1-42(mouse)} > Aβ_{1-40(mouse)}$] [11,28]. Aβ forms 3.5 metal-ion-binding sites (per subunit, as oligomers) of various affinities; the highest affinity for Zn^{2+} is ~100 nM, but that for Cu^{2+} is 8 aM [28]. This affinity is so high that it is likely that Aβ is bound to Cu^{2+} physiologically. Aβ precipitation by Cu^{2+} and Fe^{3+} is far less than that induced by Zn^{2+} at pH 7.4, yet Cu^{2+} and Fe^{3+} are also found at high levels in amyloid plaques (Zn^2, 1055 μM; Cu^{2+}, 390 μM; Fe^{3+}, 940 μM), compared with normal age-matched neuropil (Zn^{2+}, 350 μM; Cu^{2+}, 70 μM, Fe^{3+}, 340 μM) [16]. This suggests that when Aβ is precipitated by synaptic Zn, it co-precipitates with Cu^{2+} and Fe^{3+}, a possibility supported by the observation of selective Cu^{2+}- and Zn^{2+}-binding sites on Aβ [28].

The Fe in plaques is found predominantly in neuritic processes, probably complexed with ferritin [29], and might not directly interact with plaque Aβ because, unlike Cu and Zn, Fe does not co-purify with Aβ from post-mortem AD brain tissue [18]. In addition, studies of various metal-ion chelators in solubilizing Aβ from post-mortem AD-affected brain tissue have correlated the dissolution of precipitated Aβ with the release of Cu and Zn, but not Fe [7].

Cu^{2+} and Fe^{3+} interaction with Aβ mediate the toxicity of the peptide in cell culture. Aβ catalyses H_2O_2 generation through the reduction of Cu^{2+} and Fe^{3+}, using O_2 and biological reducing agents (e.g. cholesterol, vitamin C, catecholamines) as substrates [18,30,31]. Consistent with these biochemical properties being responsible for disease, it has been found that the neurotoxicity of Aβ in culture is mediated by $AβCu^{2+}$ (or $AβFe^{3+}$)

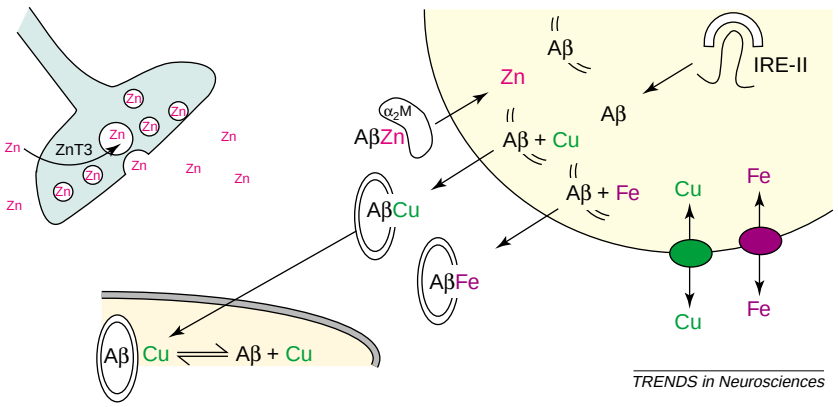

Fig. 2. Model for the non-pathogenic metallobiology of β-amyloid (Aβ). Proposed normal interactions of Aβ with brain biometals. Expression of Aβ promotes the efflux of Cu and Fe from cortical cells, and the influx of Zn [48]. Most Aβ is normally membrane associated [6,7], and membrane-inserted Aβ complexes might be the normal configuration to carry Cu^{2+} and Zn^{2+} [20,47]. The concentration of extracellular, soluble Aβ is low. With the exception of Zn^{2+} released as an exchangeable ionic form during synaptic transmission (reaching a concentration of up to 300 μM in the synapse), the metal ions are not in free ionic forms but, for simplicity, the fluxes of the metal ions are indicated without their transport or binding proteins. Zn^{2+} is taken into neurons by a transport mechanism, maintaining a low concentration of baseline extracellular Zn^{2+} (submicromolar). α_2-Macroglobulin (α_2M), a proposed risk factor for Alzheimer's disease (AD), might participate in Zn^{2+} uptake by binding Aβ (reviewed in Ref. [26]). Metal ions exteriorized by Aβ (possibly in a lipid particle) can be recycled or moved into the plasma, perhaps explaining the increase in plasma Cu levels reported in individuals with AD [62]. Translational regulation by a type-II iron-responsive element (IRE-II) might increase the availability of amyloid-precursor protein (APP) and Aβ in response to elevated cellular levels of Fe and possibly Cu [58], which could help remove the excess load of these metal ions from the cell. Aβ handles an unidentified component of cellular metal-ion metabolism in parallel to other established mechanisms for uptake and efflux of Cu and Fe.

forming H_2O_2, and, again, differs thus: $A\beta_{1-42(human)} > A\beta_{1-40(human)} \gg A\beta_{1-40(mouse)}$ [31,32]. Conversely, Aβ is not toxic in the absence of Cu^{2+} [18]. H_2O_2 is freely permeable across all tissue boundaries. Unless scavenged by defences such as catalase and glutathione peroxidase, it will react with reduced metal ions (Fe^{2+}, Cu^{+}) to generate OH· (Fenton reaction), which (in turn) generates lipid peroxidation adducts, protein carbonyl modifications and nucleic acid adducts (such as 8-OH guanosine) in various cellular compartments. Such oxidative damage typifies AD neuropathology [33–35] and precedes Aβ deposition in AD [35–37].

H_2O_2 production by Aβ is inhibited by chelators [31]. Zn^{2+} partially quenches H_2O_2 production from AβCu complexes, rescuing its toxicity in cell culture while simultaneously precipitating the peptide [18,38,39]. This effect is consistent with the observation that the quantity of H_2O_2-mediated 8-OHG adducts in neocortical tissue affected by AD is inversely proportional to plaque surface area [39]. However, Zn^{2+} suppression of H_2O_2 production by AβCu is inefficient [18], and the plaque amyloid, which contains Cu^{2+} and Fe^{3+}, is very abundant. Therefore, levels of 8-OH guanosine adducts are still markedly elevated in individuals with AD compared with age-matched control tissue [39,40]. These findings suggest that plaque β-amyloid, although conspicuous, is not as toxic as soluble or diffuse forms of Aβ, which are probably not bound to Zn^{2+}. Indeed, soluble (but not insoluble or plaque) brain Aβ levels correlate with advanced neuropathological changes such as neuritic pathology and neurofibrillary tangles [6,41,42].

The simultaneous generation by Aβ of Cu^{+} or Fe^{2+} with H_2O_2 makes the peptide vulnerable to OH· attack [30]. Aβ is markedly vulnerable to crosslinking and oxidation induced by Cu^{2+}, which leads to side-chain damage (especially of histidine), and sodium dodecyl sulphate (SDS)-resistant oligomerization [28,43]. SDS-resistant oligomerization of Aβ enhances its neurotoxicity [44,45], and it will be important to determine whether the mechanism of this modification in AD is Cu-mediated. Oxidative oligomers are resistant to proteolysis [46]; therefore, oxidized Aβ oligomers are likely to become subunits for Zn^{2+}-induced assembly into the amyloid mass (Fig. 3).

One unexplained issue is why synaptic Zn^{2+} begins to precipitate Aβ with age. Although fatigue of energy-dependent Zn^{2+}-uptake mechanisms (involving ZnT3) is one possible mechanism, another possibility is the rise in soluble Aβ in the synaptic vicinity [6,7]. Soluble Aβ is not detected in healthy brain, where it is predominantly membrane associated [6,7]. The rise in brain soluble Aβ levels in individuals with AD suggests that Aβ could be abnormally liberated from the membrane. Cu^{2+} and Zn^{2+} facilitate the integration of Aβ as hexameric complex into lipid membranes [20,47], which we hypothesize might reflect the normal site of interaction of these metal ions with Aβ. We hypothesize that adverse effects such as precipitation and neurotoxic H_2O_2 production occur only when Aβ is not inserted correctly into membrane, or is liberated to form soluble Aβ (which itself could be in a crosslinked or oligomerized state). This could occur if Aβ becomes oxidized within the membrane, perhaps as a result of age-associated increases in brain Cu and Fe levels [48] (Fig. 3). H_2O_2 signals the generation of Aβ in cell cultures [49] and it will be important to determine whether this Aβ is in the membrane or oxidized. As the disease progresses, H_2O_2 originating from increased Cu^{2+} and Fe^{3+} levels in tangle-bearing neurons might also become a source of oxidation [40].

The biochemical compartments within the brain that are affected by the age-dependent rise in Cu and Fe [48], and the cause of this elevation, remain to be elucidated. A consensus has emerged in the literature that Cu, Fe and Zn are found at markedly high levels in amyloid plaques; however, there are conflicting reports about changes in cortical tissue metal-ion concentrations in AD (reviewed

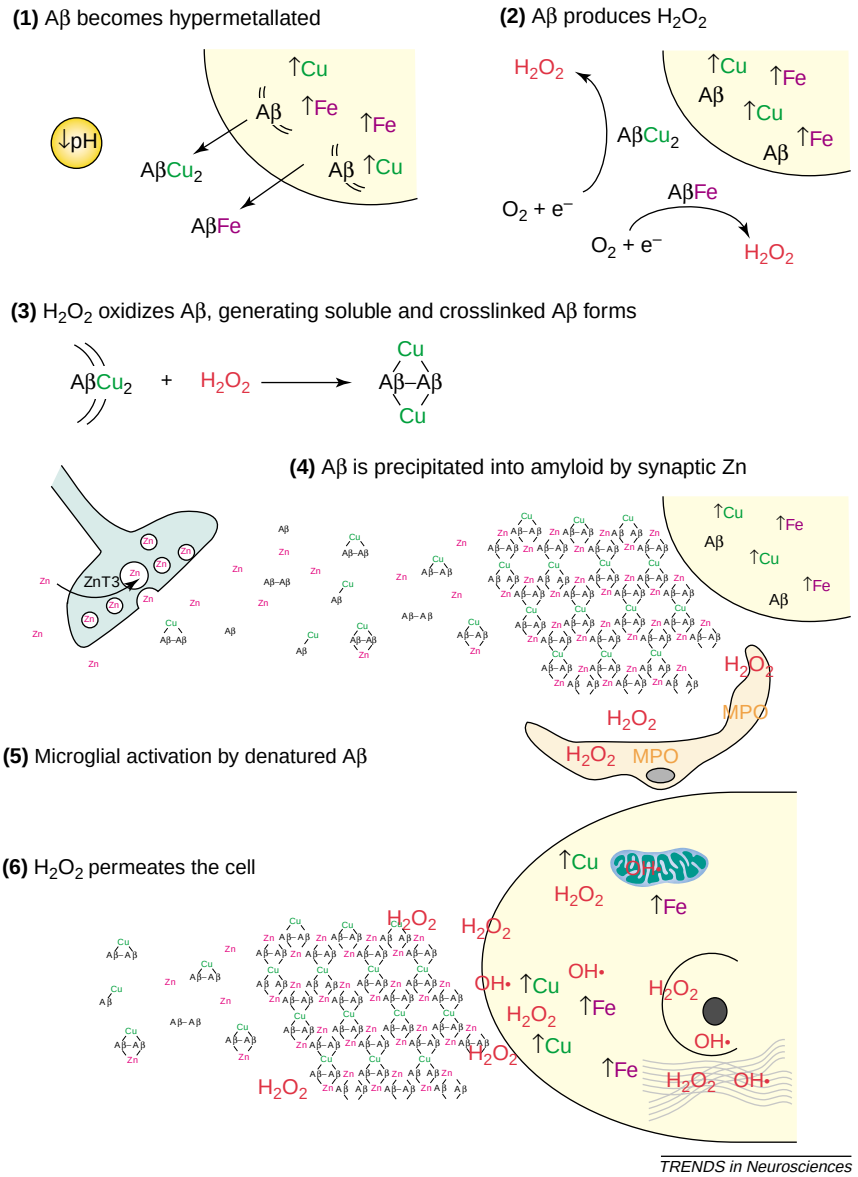

Fig. 3. Model for the metallobiology of β-amyloid (Aβ) in Alzheimer's disease (AD). Proposed sequence of biochemical events leading to AD. (1) The concentrations of Fe and Cu rise with increasing age in the brain cortex [48]. This leads to an overproduction of amyloid precursor protein (APP) and Aβ in an attempt to suppress cellular metal-ion levels [58]. If metal-ion levels continue to rise, hypermetallation of Aβ occurs, facilitated by mild acidosis [11,28]. (2) Some forms of hypermetallated Aβ catalytically produce H_2O_2 from O_2 and biological reducing agents [18]. (3) AβCu reacts with H_2O_2 to generate oxidized and crosslinked forms that are liberated from the membrane. Oxidation of Aβ makes it protease resistant. (4) These oxidized forms of Aβ are the major components of plaque deposits [77]. The release of soluble Aβ presents the peptide for precipitation by the high concentrations of Zn released in the synaptic vicinity. Plaques are therefore an admixture of Aβ with high concentrations of Zn, Cu and Fe. (5) The oxidized Aβ initiates microglial activation. The microglia characteristically react by producing high concentrations of H_2O_2 and myeloperoxidase (MPO) [78], which fosters further crosslinking of Aβ and H_2O_2 build-up outside the cortical cells. (6) H_2O_2 is freely permeable across lipid boundaries and it crosses from the outside of the cell into cellular compartments, where it reacts with Cu and Fe (the levels of these having been elevated by age), causing the production of the highly reactive hydroxyl radical (OH·), and the oxidation of nucleic acids, proteins and lipids that characterize AD-affected brain tissue. The oxidation of tau could lead to its aggregation and the development of further adventitious redox-active metal-ion-binding sites within the cell (not shown) [40].

in Refs [1,2]). For example, whereas elevated Zn is consistently observed in plaques, there are also reports of Zn deficiency in interstitial cells. The corruption of Aβ might therefore be associated with an abnormal distribution of metal ions, rather than a bulk elevation of tissue concentrations. Metal ions in the brain are handled by numerous protein and transport systems, which have been reported to be abnormal in individuals with AD, as well as the genetic risk factor proteins apolipoprotein E and α_2 macroglobulin (reviewed in Refs [1,2]). There is no conclusion yet about which of these abnormalities are more important in the cascade of events that lead to abnormal binding of metal ions to Aβ. Among reported disturbances of the most generic systems that handle metal ions in the brain are metallothionein deficiencies in brain tissue from individuals with AD (reviewed in Ref. [1]). Failure of incorporation of Zn^{2+} or other metal ions into the metallothionein system could contribute to pooling of metal ions in the extracellular space and contribute to Aβ precipitation. A further means of controlling metal-ion levels in the brain is the blood–brain barrier (BBB), which resists the transduction of fluctuating levels of plasma metal ions [50,51].

Numerous abnormalities of the BBB have been reported in individuals with AD [52,53], which could foster a rise in metal-ion levels in the cerebrovascular vicinity and contribute to amyloid deposition.

Aβ and APP might function as a metal-ion clearance system

The most commonly held view is that Aβ itself is a junk peptide. However, as its precursor is highly abundant, and because Aβ itself is generated from APP by a sophisticated proteolytic system and then rapidly turned over, the possibility that Aβ participates in biological function warrants consideration. We have hypothesized that Aβ and APP become corrupted in the biochemistry of their functions: to participate in metal-ion homeostasis and to control metal-ion-mediated oxidation.

APP possesses Cu- and Zn-binding sites in its N-terminal ectodomain, which modulate the adhesivity of the protein [54], the inhibitory effects of the KPI domain [55] and the transfer of Cu^{+} [56]. Aβ represents a second metal-ion-binding domain on APP. The exceptionally high affinity (attomolar) $A\beta_{1-42}$ has for Cu^{2+}, the selectivity of the Cu^{2+}- and Zn^{2+}-binding sites [28], the ability to bind 3.5 moles of Cu^{2+} or Zn^{2+} per mole of peptide [28], and the positively cooperative nature of Cu^{2+} binding [20] are compatible with physiological purposeful metal-ion binding. Indeed, Cu^{2+} would co-compartmentalize with the peptide in the acidic endosome during synthesis, a compartment where Cu^{2+} is loaded into superoxide dismutase 1 (SOD1).

Several lines of recent data support a role for APP and Aβ in modulating tissue metal-ion homeostasis. *App* knockout mice have elevated Cu levels in brain and liver [57]. Conversely, overexpression of the C-terminal fragment of APP, which contains Aβ, results in significantly reduced Cu and Fe levels in transgenic (C100) mouse brain, whereas overexpression of APP in Tg2576 transgenic mice results in significantly reduced Cu (but not Fe) levels, throughout the lifespan of the mouse [48]. The overexpression of APP and its derivatives in transgenic mice decreases metal-ion levels by approximately the same degree throughout the lifespan of the mouse, regardless of the accumulation of amyloid, arguing that Aβ or APP themselves are not the cause of the age-dependent elevation in brain Cu and Fe seen in normal mice. Rather, the upregulation of Aβ or APP might function to oppose this elevation in metal ions [48].

Supporting this functional possibility for APP and its derivatives, we recently found that the translation of APP is regulated by a novel iron-regulatory element (IRE-Type II) within the 5′-untranslated region (5′UTR) of APP [58]. The *App* mRNA IRE is located immediately upstream of an interleukin 1 (IL1)-responsive acute box domain. APP 5′UTR-conferred translation is selectively downregulated in response to intracellular chelation and upregulated by exposure to Fe. The binding of IRE to the APP 5′UTR is decreased after treatment of cells with desferrioxamine and increased after IL1 stimulation [58]. IRE-dependent pathways govern the post-transcriptional expression of many proteins involved in Fe metabolism, including ferritin and the transferrin receptor. As Aβ overexpression in CT100 transgenic mice leads to decreased brain Cu and Fe levels [48], the high-turnover APP protein system might therefore be upregulated in response to elevated cellular Cu and Fe, either to remove these metal ions from the cell or to prevent their uptake (Fig. 2).

Other recent studies suggest that Aβ could trap excess extracellular Cu and prevent it from adversely participating in radical-generating activity [59–61]. The possibility that Aβ directs Cu into the circulation is supported by recent data indicating that serum Cu is selectively and markedly elevated in individuals with AD [62]. Taken together, the biochemical behaviour of Aβ is therefore pleiotropic: at a high peptide to metal-ion stoichiometry, Aβ removes the metal ion and is protective; however, at high metal-ion-to-peptide stoichiometry, Aβ becomes aggregated and catalytically pro-oxidant.

What is still unclear is the nature of the biochemical switch that would alter the binding of Cu or Fe to Aβ, so that rather than being subtracted from further redox activity, the Aβ amplifies the redox activity of Cu and Fe [18,30,31]. Two factors that could play crucial roles in this switch are Zn^{2+} and membrane insertion. Zn^{2+} inhibits Aβ-mediated redox activity [18,30,39], whereas membrane insertion shields Met35 from redox activity [20,47].

The physiological fraction of brain metal-ion metabolism that may be handled by Aβ and/or APP has not yet been identified. However, changes in knockout and transgenic mice indicate that Aβ and/or APP might export ~15% of the bulk Cu pool in brain [48,57]. The fraction of total brain metal that interacts with Aβ and/or APP might not need to be large to be of pathophysiological significance. Reactive oxygen species are generated catalytically by trace concentrations of Cu or Fe, which necessitates stringent homeostatic control of these metal ions. Similarly, the fraction of Zn responsible for plaque amyloid deposition is only 15–30% of total brain Zn [24,26].

Pharmacological interdiction of Aβ metalloprotein reactions as the basis for novel AD therapeutics

The principle of a pharmacotherapeutic molecule complexing a metal-ion-binding site on a protein target is well developed in pharmacology, and is very different to chelation therapy. Several well-known antibiotic, anticonvulsive, anti-tumour and anti-inflammatory drugs [2] exert their pharmacological effects by interacting with the metal-ion active site of their target protein. For example, non-steroidal anti-inflammatory drugs such as diflunisal, ibuprofen and indomethacin block the haem–Fe catalytic site on cyclooxygenase [63,64].

Oral treatment with clioquinol (CQ), a retired United States Pharmacopeia antibiotic and orally bioavailable Cu–Zn chelator, induced a 49% decrease in brain Aβ deposition ($-375\ \mu g\ g^{-1}$ wet weight, $P = 0.0001$) in a blind study of Tg2576 transgenic mice treated orally for nine weeks [65]. There was no evidence of neurotoxicity, and general health and body weight parameters were significantly more stable in the treated animals, the condition of which conspicuously improved after only 16 days of treatment. Although the transgenic mice studied were different, comparison of CQ treatment with the best effects of the well-known Aβ vaccine [3] indicates that the

inhibition of cerebral Aβ deposition (in absolute amount) by CQ was more extensive, and was achieved more rapidly. Therefore, CQ treatment appears, like the vaccine therapy, to be a potent inhibitor of Aβ accumulation. CQ has now proceeded into Phase I [66] and Phase II clinical trials in individuals with AD.

Unlike common chelators such as penicillamine, CQ is hydrophobic and crosses the BBB. Indeed, treatment of the Tg2576 mice with the traditional hydrophilic Cu chelator, TETA (triethylenetetramine), did not inhibit amyloid deposition [65], indicating that systemic metal-ion depletion (e.g. 'chelation therapy') is not likely to be a useful therapeutic strategy for AD. CQ might work by a combined action that facilitates disaggregation of the Zn-mediated Aβ collections [11,67], while also inhibiting Cu- or Fe-mediated H_2O_2 production catalysed by Aβ [18,65]. H_2O_2 inhibits LRP-mediated clearance mechanisms [68]. The inhibition of metal-ion-mediated H_2O_2 production from Aβ by CQ could therefore facilitate Aβ clearance mechanisms. The resolubilized Aβ might then either be removed into the blood, as observed in Aβ-immunized transgenic mice [69], or degraded by intracellular uptake and hydrolysis. CQ is as effective as high-affinity chelators in blocking the production of H_2O_2 by Aβ *in vitro*, in preventing precipitation of synthetic Aβ by Zn^{2+} and Cu^{2+}, and in extracting Aβ from post-mortem AD brain specimens [65].

Importantly, CQ treatment did not induce a loss in metal-ion levels systemically, probably because it is a relatively weak (K_a is nanomolar for Zn^{2+} and Cu^{2+}) chelator and the metal ions are redistributed rather than excreted. Therefore, the benefits of the drug appear to be due to its ability to bind selectively to the Aβ–metal-ion complex, and are not due to metal-ion depletion of brain tissue. It is unusual that a chelator with such relatively low affinity for metal ions can be so potent at inhibiting metal-ion interactions with Aβ [65]. The hydrophobicity of CQ (and possibly other stereochemical properties) might facilitate access to the metal-ion-binding site on Aβ. However, it is also possible that the pathophysiology of AD is mediated by the low-affinity metal-ion-binding sites on Aβ. Aβ can bind up to 3.5 moles of Cu^{2+} and Zn^{2+} [28] per peptide subunit, and achieves this high degree of metallation by interstrand histidine bridges [21]. We suspect that the lower affinity sites on Aβ that modulate peptide precipitation [11,28], and are also redox active [18], might be pathological and appear when the peptide is liberated from the membrane leaving the C terminus unshielded by lipid. The affinity of CQ is sufficient to dissociate Zn^{2+} and low-affinity bound Cu^{2+} from Aβ, and we have observed CQ inducing Cu^{2+} dissociation from Aβ by NMR spectroscopy [65]. CQ does not interact directly with Aβ [65]. Therefore, CQ might be therapeutic by reacting selectively with the pathological, relatively low-affinity metal ions bound to Aβ.

CQ treatment of non-transgenic mice significantly decreases brain levels of Cu, Zn and Fe [70]. However, treatment of 21-month-old APP2576 mice with CQ for nine weeks paradoxically elevated brain Cu by 19% and Zn by 13%, while markedly inhibiting Aβ deposition. This unexpected result is illuminated by recent findings (discussed above) that brain Cu, Fe and Zn levels might be influenced by production of Aβ and/or APP, and that Aβ might play a role in trafficking Cu out of, and Zn into, the cell [48]. Metal-ion pooling in amyloid deposits could leave the neighbouring cells relatively metal-ion deficient. Therefore, the paradoxical increase in Cu and Zn in CQ-treated APP2576 mice might be explained by CQ preventing Cu^{2+} and Zn^{2+} from complexing with extracellular Aβ, and then diverting metal ions for uptake into metal-ion-deficient brain tissue, instead of being sequestered into amyloid (Fig. 4). So, despite being a chelator, CQ might be able to correct homeostatic defects of brain metal-ion metabolism that could occur in AD. Furthermore, CQ [and desferrioxamine (DFO)] might inhibit APP translation and Aβ production, but although this effect has been observed *in vitro* [58], the Tg2576 mice treated with CQ have a PrP promoter and lack the 5′UTR of APP [65].

One previous trial of DFO was reported to induce a significant slowing in the rate of progression of dementia [71]. This effect was attributed to complexing Al^{3+} but the drug also has high affinity for Zn, Cu and Fe. The administration of DFO is associated with discouraging difficulties, including systemic metal-ion depletion and the need to deliver it by twice-daily, painful intramuscular injection. In addition, DFO is a charged molecule that does not easily penetrate the BBB and is rapidly metabolized [72]. Unlike DFO, CQ is selectively targets brain metalloprotein, and is less prone to systemic metal-ion depletion.

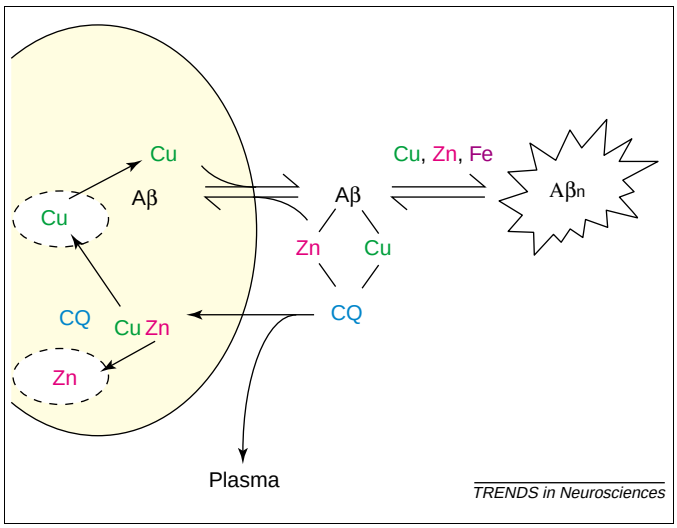

Fig. 4. Clioquinol (CQ) exerts multiple effects on β-amyloid (Aβ) metallation and metal-ion homeostasis. In bulk metal-ion analysis of aging brain, there is no net elevation in Zn levels but there are rises in those of Cu and Fe [48]. However, as Aβ deposition becomes very advanced ($Aβ_n$), these metal ions are trapped in amyloid plaques. This could lead to repartitioning, leaving neurons relatively deficient in metal ions (especially in Cu and Zn, which are released during synaptic transmission). The mechanism of action of CQ is complex. When amyloid build-up is absent or minimal, it depletes the brain of metal ions [70]. It also selectively removes Cu^{2+} and Zn^{2+} from Aβ. The affinity of CQ for metal ions is approximately nanomolar, similar to the low-affinity metal-ion-binding sites on Aβ that mediate precipitation [8,9,28] and H_2O_2 formation [18]. By competing with Aβ for these metal ions, CQ will either deplete the bulk tissue of these metal ions (passing them into the blood) or will make them available again for re-uptake into neurons that are deficient in metal ions. This could explain why Cu and Zn levels in the brain rise paradoxically after CQ-treatment of Tg2576 mice with advanced cerebral amyloid pathology [65].

Table 1. Neurodegenerative diseases in which metal interactions might mediate protein aggregation[a]

Specific protein	Metal	Specific tissue	Specific disease	Refs
Aβ	Cu, Fe, Zn	Neocortex	Alzheimer's disease	[14]
PrP^c	Cu	Neocortex	Creutzfeldt–Jakob disease	[73]
SOD1	Cu, loss of Zn	Motoneuron	Familial ALS	[74]
α-Crystallin	Cu, Fe	Lens	Cataracts	[75]
α-Synuclein	Cu, Fe	Basal ganglia	Parkinson's disease	[76]

[a]Neurodegenerative diseases in which abnormal metal-ion interaction with a protein target has been proposed as a neurochemical denominator [27]. Abbreviations: Aβ, β-amyloid; ALS, amyotrophic lateral sclerosis; PrP^c, cellular prion protein.

Concluding remarks

Characterization of the metalloprotein biochemistry of Aβ has been useful for understanding the neurochemical factors in human ageing that cause its corruption and conversion into a toxic principle in AD. The metal-ion-binding sites on Aβ provide a very promising target for the development of new therapeutics. Future research will determine whether the principles of medicinal metallochemistry in AD could possibly generalize to abnormal protein–metal-ion interactions described for other age-dependent neurodegenerative disorders such as Parkinson's disease, motoneuron disease and cataracts [27] (Table 1).

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