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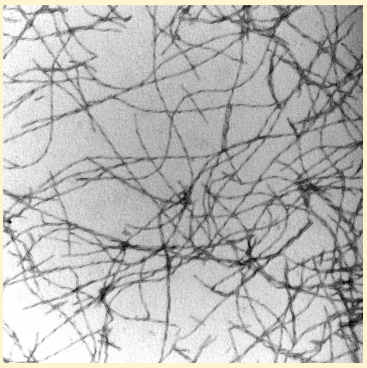
Copper and Oxidative Stress in the Pathogenesis of Alzheimer's Disease

Gözde Eskici and Paul H. Axelsen*

Departments of Pharmacology, Biochemistry and Biophysics, and Medicine, University of Pennsylvania, Perelman School of Medicine, Philadelphia, Pennsylvania 19104, United States

ABSTRACT: Copper is a redox-active metal with many important biological roles. Consequently, its distribution and oxidation state are subject to stringent regulation. A large body of clinicopathological, circumstantial, and epidemiological evidence suggests that the dysregulation of copper is intimately involved in the pathogenesis of Alzheimer's disease. Other light transition metals such as iron and zinc may affect copper regulation by competing for copper binding sites and transporters. Therapeutic interventions targeting the regulation of copper are promising, but large gaps in our understanding of copper biochemistry, amyloidogenesis, and the nature of oxidative stress in the brain must be addressed.



Metals have long been suspected to have a role in the pathogenesis of Alzheimer's disease (AD), particularly heavy metals such as lead,[1] cadmium,[2] and mercury[3] that are notoriously neurotoxic and have no biological function. Aluminum has also been suspected because of its prevalence in our environment and various pathological[4] and epidemiological[5] correlations. However, most investigators are now focused on biologically important metals such as iron (Fe), zinc (Zn), and copper (Cu) because they appear to be dysregulated in AD. Among these, copper has attracted the most attention because the amyloid precursor protein (APP) and the amyloid β (Aβ) peptides that are derived from APP both have significant interactions with copper.

This Current Topic examines the relationships among copper, oxidative stress, and Alzheimer's disease with the aim of highlighting consensus and controversy in the primary literature while avoiding the pitfalls of citation bias.[6] Although these topics have already been reviewed many times,[7−28] a fresh and comprehensive examination of this literature is needed for perspective on the diverse mechanisms by which copper dysregulation may be linked to AD pathogenesis through oxidative stress.

■ COPPER INTAKE AND TRANSPORT

Many important enzymes such as ceruloplasmin, cytochrome *c* oxidase, dopamine β-hydroxylase, superoxide dismutase, lysyl oxidase, and tyrosinase rely on copper for their catalytic activity.[29] Consequently, a dietary deficiency of copper has diverse manifestations,[30] and some have suggested that a copper deficiency contributes to the pathogenesis of AD.[31,32] A dietary excess of copper has no obvious short-term consequences because copper homeostasis can be maintained by gastrointestinal and liver transporters that control its absorption and elimination. However, AD-like pathology has been induced by excess dietary copper in rabbits on a high-cholesterol diet,[33] and in a mouse model of AD.[34] Observations of this nature, combined with reports that copper levels in various tissues and fluids may be elevated in AD (reviewed below), have prompted suggestions that chronic exposure to excess copper (e.g., via copper plumbing) contributes to AD in humans.[35] Taken altogether, the evidence for a simple relationship between AD and dietary copper intake is decidedly mixed, and far from compelling in either direction.[36] If copper has a significant role in AD pathogenesis, the relationship is likely to depend in a complex way on the transport, distribution, or chemical interactions of copper in microenvironments of the human brain, rather than in any simple way on the level of dietary intake.

The transport of copper from the digestive tract into gut epithelium, and from portal venous blood into hepatic cells, is mediated chiefly by copper plasma membrane transporter 1 (CTR1).[37−39] Surprisingly small amounts of dietary zinc compete for transport by CRT1 in the gut and can cause copper deficiency.[40] Prior to uptake and transport by CTR1, Cu(II) ions are reduced to Cu(I) by membrane-bound metalloreductases.[17,41] Within hepatic cells, chaperone proteins deliver Cu(I) to copper-dependent enzymes, or to transport systems.[42] ATOX1 is a chaperone that delivers Cu(I) ions to ATP7A, a P-type ATPase transporter in intestinal epithelial cells that exports copper either into the portal vein or back across the luminal membrane for elimination from the body.[43]

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The primary transporter in the liver is ATP7B, which releases some hepatic copper into the bile for elimination from the body and some of it into the blood where it is transported by albumin and, presumably, by human analogues of transcuprein-like macroglobulins that have been identified in the rat.[44] Subtle mutations in the ATP7B gene are common and may be a genetic factor that increases blood copper levels and the risk of AD.[45] ATP7B is also responsible for exporting hepatic copper into ceruloplasmin, a plasma protein that is synthesized mainly in the liver and normally contains six Cu(II) ions.[46] Although 95% of the copper in plasma is found in ceruloplasmin, the copper atoms do not exchange over time, indicating that it does not function as a copper transporter.[47] Instead, the primary function of ceruloplasmin appears to be related to the oxidation of various substrates, particularly Fe(II), to the four-electron reduction of water.[47,48] The oxidation of Fe(II) to Fe(III) occurs spontaneously under physiological conditions, but the involvement of ceruloplasmin may minimize the toxicity of the electron that is elaborated.[48]

The brain has multiple mechanisms for the uptake of copper from circulating albumin and low-molecular weight copper complexes,[49,50] as well as for the uptake of free copper ions across the blood–brain barrier.[51] Cerebrovascular endothelial cells and perivascular astrocytes, which largely comprise the blood–brain barrier, both take up copper via ATP7A, while the latter cell type can take up and store considerable amounts of copper[52] even when extracellular concentrations are low.[53] Hspa5 is a chaperone that is specifically induced by copper in neonatal rat astrocytes and involved in the regulation of their accumulation of copper.[53]

Ceruloplasmin does not cross the blood–brain barrier, but a substantial amount of GPI-linked ceruloplasmin is produced within the central nervous system, chiefly in the substantia nigra, retina, Schwann cells, and perivascular astrocytes.[54−58] In the congenital absence of ceruloplasmin (aceruloplasminemia), degeneration of the basal ganglia occurs with evidence of iron overload.[59,60] Serum ceruloplasmin levels are reportedly unchanged in AD,[61] but ceruloplasmin levels in the brain are broadly increased.[62] The level of apo-ceruloplasmin (ceruloplasmin lacking a full complement of copper ions) may be elevated in AD,[63] along with the amount of exchangeable or "labile" copper in the tissues.[64]

At glutamatergic nerve terminals, copper accumulates in synaptic vesicles and is released along with zinc and neurotransmitters upon depolarization.[65−67] The oxidation state of the released copper is uncertain. One of the indicators used to demonstrate this release is presumably specific for Cu(II), but it cannot distinguish copper that is released as Cu(II) from copper that is released as Cu(I) and rapidly oxidized upon release.[68] In either case, copper released into the synaptic cleft may be taken up by high-affinity mechanisms that have been observed in synaptosomes.[69] Because of the small dimensions of the cleft, released copper may reach concentrations as high as 100 μM.[66]

The physiological role for copper in this setting is unclear, but a hint may be that the availability of copper for release at nerve terminals depends on the movement of an ATP7A-like transporter from somatic Golgi membranes to the nerve terminals, movement that is stimulated by NMDA receptor activation[70] and protects against glutamatergic excitotoxicity.[71] Protection requires endogenous nitric oxide production, suggesting that the protective mechanism may involve copper-mediated reaction of nitric oxide with thiols, and possibly the S-nitrosylation of NMDA receptors. The latter is a well-documented mechanism for downmodulating their function.[72] Alternatively, copper may bind to the prion protein, PrP^C, and thereby regulate the activity of NMDA receptors.[73]

COPPER AND NEUROPATHOLOGY

Mutations in ATP7A and ATP7B lead to well-known diseases that highlight the roles of these proteins in copper transport.[74] Mutations in ATP7A cause Menkes disease, characterized by impaired absorption of copper from the gut lumen into epithelial cells and impaired export from those cells into the portal system. Consequently, copper accumulates in tissues such as the small intestine and kidneys, while the brain and other tissues have unusually low levels of copper and reduced activity of copper-dependent enzymes. Mutations in ATP7B cause Wilson's disease, characterized by the accumulation of copper in the liver.[30] Wilson's disease may be treated by oral zinc administration, which promotes the elimination of copper via the stools by competing for copper uptake by CRT1, and inducing the production of metallothionein, a protein that appears to store copper in the gut mucosa.[75]

Both Menkes disease and Wilson's disease have prominent central nervous system manifestations, although the distribution of neurodegenerative changes and the histological appearance of these changes are distinct from those associated with AD. Menkes disease manifests primarily in the cerebellum, while Wilson's disease primarily affects the basal ganglia. AD can manifest in both of these regions, but it primarily affects the cortical gray matter and is characterized by amyloid plaques and neurofibrillary tangles. Neither of these histopathological features is prominent in Menkes disease or Wilson's disease. Nevertheless, the role of impaired copper transport in the central nervous system and neurodegeneration in these diseases has fueled interest in the possible role of copper dysregulation in the pathogenesis of AD.

If AD is due to a copper transport disorder, the concentration, distribution, or turnover of copper should be altered in a relevant tissue compartment. Occasional studies have reported that serum or plasma copper levels are lower in AD,[76,77] but most have reported that levels are unchanged[63,78−83] or slightly higher[61,84−89] than control groups. Elevations of levels of free (i.e., exchangeable, not bound to ceruloplasmin) copper in AD have been noted in some studies.[63,88,89] Copper levels in the cerebrospinal fluid (CSF) vary widely; some have reported them to be increased in AD,[90,91] while others report that the variance in a population obscures any apparent difference between normal and AD.[61,79,92]

In human brain tissue, most reports have concluded that copper levels are either unchanged or slightly lower in regions affected by AD.[93−99] An X-ray fluorescence imaging study found increases in the overall copper concentration in the hippocampus of transgenic mice between 3 and 12 months of age.[100] However, an increased concentration in control mice at 18 months of age eliminated this difference, and no differences were detected in cortical brain.

On a microscopic level, X-ray fluorescence microprobe studies have demonstrated that deposits of copper and zinc colocalize with amyloid plaques in human AD brain tissue.[101,102] ICP-MS studies[103] of human samples isolated by laser capture microdissection[104] and proton microprobe studies[105] also reached this conclusion. In transgenic PSAPP mice, however, an X-ray fluorescence microprobe study found

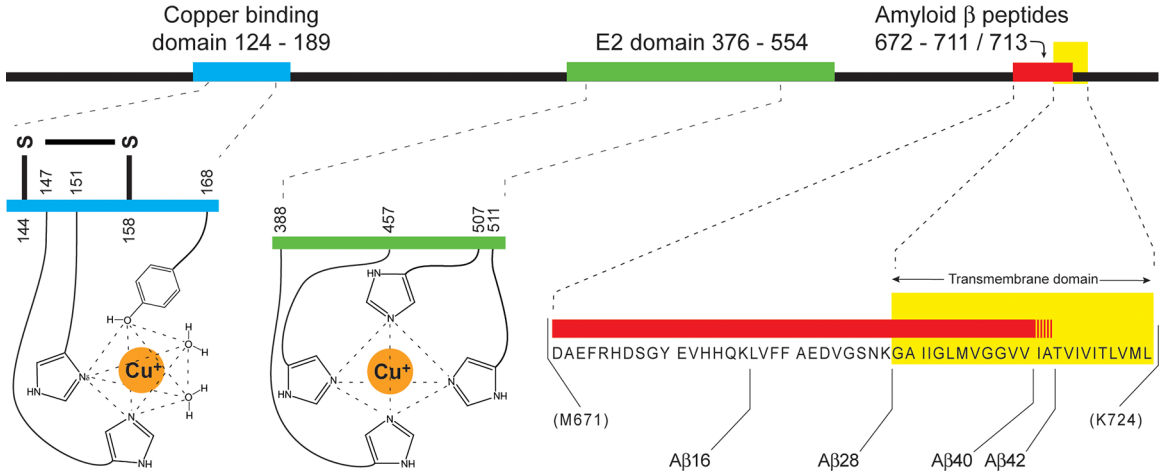

Figure 1. A schematic illustration of the 770-residue APP sequence (black) showing the copper-binding domain (blue), E2 domain (green), Aβ peptides (red), and the α-helical transmembrane domain (yellow) within the primary sequence. All residue numbering is according to the APP770 isoform. The copper-binding domain is expanded to illustrate the primary sequence locations of residues His147, His151, and Tyr168, which form (with two waters) a five-coordinate binding site for Cu(II).[110] Reduction to Cu(I) is accompanied by disulfide bond formation between residues Cys144 and Cys158. The E2 domain is expanded to illustrate the primary sequence locations of residues His388, His457, His507, and His511, which form a four-coordinate copper-binding site.[111] The Aβ–transmembrane region is expanded to illustrate the amino acid sequences of Aβ16, Aβ28, Aβ40, and Aβ42. The numbering of residues in Aβ peptides corresponds to the numbering in APP minus 671. Small amounts of Aβ39 and Aβ43 are also found in AD brain, as well as various N-terminal truncations including pyroglutamate-3. An α-secretase activity cleaves APP at the C-terminal end of Aβ16 (i.e. after Lys687) and commits APP degradation to the so-called non-amyloidogenic pathway. The amyloidogenic pathway leading to the production of Aβ peptides involves β-secretase activity cleaving after Met671, and γ-secretase activity cleaving at various points within the helical transmembrane domain.

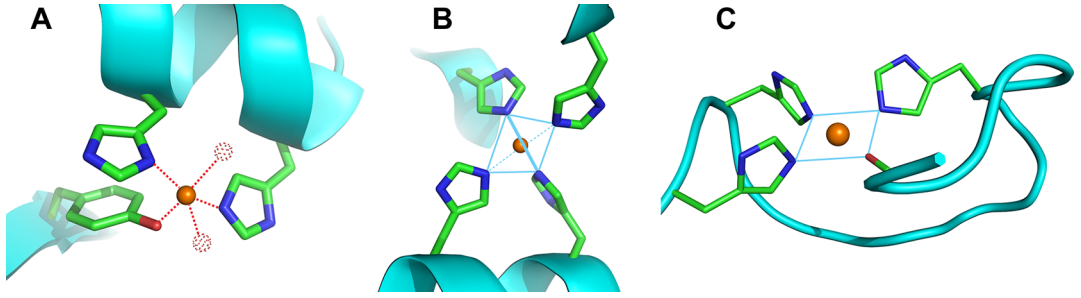

Figure 2. Copper coordination geometries in APP and Aβ. A: Five-coordinate geometry in a crystal structure of the APP copper-binding domain (PDB:2FK1).[110] B: The "tetrahedrally distorted square planar" four-coordinate geometry of the copper-binding site in a crystal structure of the APP E2 domain (PDB:3UMK).[111] C: Square planar coordination geometry in a model of Aβ16 created in silico with the backbone carbonyl oxygen of Ala2 and the ε-nitrogens of His6, His13, and His14 as ligands. In all three panels, the copper atom (orange) is rendered with a surface drawn at 25% of its ionic radius. Images prepared using PyMol (Delano Scientific, San Carlos, CA).

that the concentration of copper in amyloid plaques was lower than that in the surrounding tissue.[106] A possible explanation for differences in the copper content of plaques in human and transgenic mouse brain is that plaques in the latter contain both wild-type murine and human forms of the Aβ peptides.[107,108]

The analysis of metal ion concentrations in brain tissue is complicated by sample preparation requirements, which can involve multiple steps, and the possibility of contamination at any of these steps. For some techniques, standards in a matrix comparable to brain tissue are not available. As the size of the analyzed volume becomes smaller and more narrowly focused on the amyloid plaque, the lack of control samples from brains that do not have amyloid plaques becomes problematic. Yet another complication is the fact that the most sensitive techniques do not distinguish among the various physicochemical states in which copper may exist.

In summary, disorders of copper transport are clearly capable of causing neurodegenerative disease, but the pathology of AD differs in many fundamental ways from the pathology of diseases for which genetic origins of transporter dysfunction have been identified. It remains to be seen whether AD is merely a different kind of copper transport disorder. As this question is pursued, it is difficult to draw inferences about the nature of such a transport disorder from altered copper levels in the serum, CSF, or tissue alone. More information is needed about the physical state and subcellular distribution of copper within the affected tissues.

■ COPPER AND APP

APP is synthesized as a 770-residue multidomain transmembrane protein, of which residues 1–17 are a signal peptide. Within the 753 residues remaining after signal processing, there are several recognizable domains, including a transmembrane helix spanning residues 700–723 and two copper-binding domains (Figure 1).[12] The region in APP that gives rise to Aβ peptides is not known to interact with copper until after it has been enzymatically cleaved from APP. After being liberated from APP, however, Aβ peptides exhibit a

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strong in vitro affinity for copper. Copper binding affects the rate and paths by which Aβ peptides form amyloid fibrils and plaques, the latter being one of two characteristic histopathological lesions in AD. The other characteristic lesion is the neurofibrillary tangle, composed of hyperphosphorylated tau protein, which may also interact with copper. It remains unclear whether these protein aggregates are part of the pathogenic mechanism of disease or byproducts of the disease process. Nevertheless, their consistent appearance in AD compels us to investigate how they form and how copper is involved, if only to gather clues about an antecedent process that may be more directly responsible for disease pathogenesis.

Nuclear magnetic resonance (NMR) and crystallographic studies of the isolated copper-binding domain (CuBD), suggest that the APP binds copper with a non-planar coordination geometry that favors Cu(I) coordination (Figure 2a).[109,110] A crystallographic study of the E2 domain identified an altermative higher affinity site with "tetrahedrally distorted square planar" geometry (Figure 2b), noting that copper-binding induced a significant structural rearrangement within APP.[111] In the CuBD, Cu(II) is reduced to Cu(I) upon binding to APP,[112] possibly to some extent because Cu(II) ions ordinarily favor square planar coordination.[113] Cu(II) reduction is accompanied by the formation of a disulfide bridge between residues 144 and 158,[114,115] which confers site-specific vulnerability to peroxide and results in fragmentation of the protein.[114]

Diverse functions have been attributed to APP, including a ceruloplasmin-like iron export and ferridoxase activity[116] that responds to elevated intracellular iron levels through a genetic response element.[117] While its primary role in normal physiology remains unclear,[118,119] and there is little or no similarity between the copper binding site of APP and the binding sites of known copper transporters,[111] several lines of reasoning and evidence suggest that APP also has a role in the transmembrane transport of copper. For example, despite the dissimilarity, the reduction of Cu(II) to Cu(I) by APP upon binding is typical of other copper transporters. Moreover, cells respond to copper and APP manipulations in a manner that is consistent with this role: copper deficiency reduces APP mRNA levels,[120] while copper loading increases them.[121] Copper loading also promotes the net movement of APP from intracellular locations to the surface of cultured neuronal cells[122] and increases cellular APP levels.[123] Intracellular copper depletion by overexpression of ATP7A downregulates APP expression.[124] Brain tissue levels of copper are higher in APP-knockout mice[125] and lower in APP-overexpressing mice.[126,127] A divalent metal transporter colocalizes with Aβ plaques in post-mortem AD brain.[128] Silencing the expression of this transporter in cell culture reduces the level of APP expression and Aβ secretion.

Compared to APP, relatively little attention has been directed toward the interaction of copper with tau, the microtubule-associated protein that is hyperphosphorylated by GSK-3 in AD and that aggregates to form neurofibrillary tangles. Cu(II) binds to pseudorepeats[129,130] or isolated segments[131,132] of the tau protein and may be reduced to Cu(I) with the production of peroxide while bound to tau.[133] In primary neuronal cultures, copper chelation reduces PKC activity and increases GSK-3 activity, leading to aberrant tau phosphorylation.[134] Synthetic copper ligands can penetrate cells, increase intracellular copper concentrations, and inhibit GSK-3 via akt signaling.[135−137]

■ COPPER AND Aβ PEPTIDES

The nonamyloidogenic pathway for APP processing involves α-secretase activity, which cleaves APP after Lys687 in the midst of the segment that would otherwise give rise to an Aβ peptide (Figure 1). The amyloidogenic pathway, in contrast, is a product of β-secretase activity, which cleaves after residue Met671 (an extracellular site), and γ-secretase activity, which cleaves at various sites within the transmembrane helix to yield Aβ peptides with 39−43 residues. Copper is one of the many factors that influence the relative fluxes through these two pathways,[138] and it appears to increase the extent of processing through the nonamyloidogenic pathway.[120,123] Copper levels do not appear to alter β-secretase activity directly,[2] but there are copper binding proteins that may allow copper to exert indirect effects on β-secretase expression and activity.[139]

β- and γ-secretases are popular targets of pharmacological intervention because inhibiting them should reduce the level of production of Aβ peptides and, in turn, the formation of amyloid fibrils in the brain. The obvious concern with this approach is that these enzymes may have another function that is not yet entirely defined. Even the primary site of degradation is not completely defined. APP is a cell-surface protein, but the amyloidogenic processing of APP may be an intracellular process that follows endocytosis.[140] Nonspecific membrane-bound enzymes or metal-catalyzed oxidation processes may also act on APP to yield Aβ peptides.[114,141,142]

Copper ions released at glutamatergic synapses in the hippocampus[143] may bind and oxidatively modify Aβ peptides,[144,145] ultimately remaining bound in the core of an amyloid plaque. Copper may also regulate the degradation of Aβ peptides. For example, the treatment of APP-expressing CHO and neuroblastoma cells with synthetic complexes containing Cu(II) or Zn(II) can raise intracellular ion levels, activate signaling cascade enzymes, and upregulate matrix metalloproteinases that degrade Aβ peptides, ultimately decreasing their extracellular concentrations.[135,146]

Widely disparate results have been published for the affinity of Cu(II) for monomeric,[147−154] truncated,[149,155−161] and fibrillar[148] Aβ. An attempt to reconcile these disparate results using isothermal titration calorimetry concluded that the best K_a value for Aβ40 was 2.4×10^9 M^{-1} at pH 7.4, that interference due to HEPES buffer was minimal, and that all of the residues involved in copper binding were among the first 16 residues in Aβ40 because the affinity of Aβ16 was indistinguishable from that of Aβ40.[162] About the same time, a metal-binding fluorescent dye approach yielded a somewhat lower result, 2.9×10^7 M^{-1}.[163] More recently, a study using two independent spectroscopic methods (fluorescence quenching and circular dichroism) with various competing ligands at pH 7.4 concluded that the affinities of Aβ40 and Aβ42 for copper were similar to each other, and much higher, approximately 10^{11} M^{-1}.[164]

A recent isothermal titration calorimetry study of Aβ28 reported a 1:1 binding stoichiometry and a K_a of 5.8×10^9,[165] in agreement with the aforementioned K_a for Aβ40, but somewhat lower than previously reported spectroscopically determined values for this fragment.[164] The remarkable observation in this calorimetric study was that both the enthalpy and entropy of binding were increased in His → Ala variants, indicating that enthalpy−entropy compensation was responsible for the lack of change in affinity. These findings, usually interpreted as evidence of a multiplicity of weak

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interactions in a protein,[166,167] were interpreted in this case as evidence of binding plasticity within copper·Aβ28 complexes.[164]

Murine Aβ peptides have three substitutions compared to their human analogues (R5G, Y10F, and H13R), and they do not form amyloid plaque pathology in the absence of human transgene products.[168] It has been reported that murine Aβ16 has a lower affinity for Cu(I)[169] and Cu(II)[155] than human Aβ16, which is not surprising given that a likely coordination site (His13) has been replaced with a cationic side chain (Arg). The picture is unclear, however, because a recent study of full-length Aβ40 found that the murine Aβ bound Cu(II) somewhat more strongly.[170]

The affinity of Aβ peptides for Cu(I) has been estimated by one group to be $\sim 2 \times 10^7$ M^{-1} for Aβ42[169] and another to be $\sim 5 \times 10^{14}$ M^{-1} for Aβ16.[171] Compared to that for copper, the affinity of Aβ peptides for iron and zinc is lower by as much as 1 or 2 orders of magnitude,[147,163] and their binding appears to have structural consequences on Aβ peptides that differ from that of copper binding.[172]

Accurate measurements of affinity are made difficult by many factors, including the presence of trace copper in virtually all experimental circumstances, and uncertain affinities of copper for various competing ligands. Other confounding factors may include nonspecific interactions between copper and Aβ peptides, interactions with buffer components, uncertain or copper-induced changes in the aggregation states of the peptide, variable binding stoichiometries, a possible time dependence or aging of the binding site, oxidation and reduction, and changes in pH. For example, binding of copper to Aβ40 increases its isoelectric point (pI = 5.3) and causes self-association of Aβ40·Cu(II) complexes at pH 7.4.[173] Recently, much-needed emphasis has been placed on distinguishing among distinct complex species with multiple possible binding sites,[174] but the picture remains unsettled.

Conclusions about the stoichiometry of formation of the copper·Aβ complex have yielded values of 1:2 and 2:1 for high- and low-affinity sites, respectively, in full-length Aβ40 and Aβ42.[151] Fractional stoichiometries within this range may be due to the creation of additional binding sites in oligomeric forms. The presence of two sites with significantly different affinities has also been suggested for Aβ16[158] and Aβ28.[156] When more than one metal ion is bound per peptide, the preference of Aβ40 for copper and zinc is similar at pH 7.4, but the preference for copper predominates when the pH is lowered to 6.6.[151] A recent study concluded that a low-affinity second binding site can be detected in Aβ28, but that this second site is exposed in full-length Aβ42 only by methanol treatment.[164] Despite all these reports of more complex stoichiometries, most investigators have concluded that Aβ peptides and their N-terminal fragments bind copper ions in a 1:1 ratio.[147,149,152,153,155,159,162,163]

Cu(II) binding induces a biphasic change in the fluorescence intensity of Tyr10 in Aβ16, which has been used to characterize the kinetics of formation of the copper·Aβ complex.[175] At a peptide concentration of 40 μM, binding kinetics are complicated by the formation of ternary Aβ·copper·Aβ complexes. Moreover, even when the copper:Aβ ratio is only 1:10, the His6 residue in virtually all of the Aβ molecules has coordinated to copper during the time interval of the measurement, implying rapid exchange among Cu−His6 pairs. Nevertheless, complex formation is reportedly complete in less than 100 ms.

The analysis of copper·Aβ complexes by electrospray ionization mass spectrometry has indicated that Cu(II) forms 1:1 complexes with Aβ16,[158] Aβ28,[159] and Aβ40.[176] Surprisingly, Aβ(25−35) also appears to form copper complexes despite having no His residues.[177] Mass spectrometric analysis has found that Cu(II) forms a 1:1 complex with Aβ40 at pH 6.6, but that as many as two additional copper ions bind at pH 8.0.[178] Special techniques involving copper electrodes and the presence of ascorbate have been employed to generate Cu(I)·Aβ16 complexes.[179] As mentioned above, mass spectrometry imaging techniques have been informative with respect to the distribution and quantity of copper in human brain tissue.[103−105] Mass spectrometric analysis was key to demonstrating that Cu(I) and Cu(II) inhibited the degradation of Aβ16 and Aβ(16−28) by insulin-degrading enzyme.[180]

■ COPPER AND Aβ AGGREGATION

The formation of fibrils by Aβ peptides is a nucleation-dependent process, exhibiting a concentration-dependent lag phase before fibril formation begins and while the requisite nuclei form. This lag phase may be eliminated by introducing preformed fibril fragments to serve as nuclei.[181,182] Solid state NMR studies have indicated that the internal structure of amyloid fibrils includes two in-register parallel β sheets formed by residues 12−24 and 30−40.[183] Hydrogen exchange studies generally agree but suggest that the extent of these sheets may not be sharply defined.[184,185] Other features of the internal structure are poorly understood and may vary with fibrillization conditions.[186,187]

Fibrils reportedly bind Cu(II) ions with approximately the same affinity as monomeric Aβ42, in a 1:1 molar ratio, with little change in peptide secondary structure, and with the same coordination mode(s).[164] If His13 and His14 are both involved in coordinating with the copper, these observations imply that His13 and His14 are not part of a parallel β sheet because being adjacent in sequence, they would be on opposite sides of any such sheet and unable to interact simultaneously with a copper ion.

The trace amounts of copper, iron, and zinc present under most laboratory conditions may help nucleate Aβ aggregation.[188] However, with increasing copper:Aβ ratios, the aggregation pathway changes,[189−192] and the aggregating peptide is diverted into nonfibrillar forms.[176,193−198] Nonfibrillar aggregates have been known variously as protofibrils,[199−201] micelles,[202−204] ADDLs,[205] paranuclei,[206,207] or simply soluble oligomers.[208−210] Some of these nonfibrillar aggregates may be laboratory artifacts,[211] but there is little doubt that true oligomeric forms exist and that some,[212−220] though not all,[221] may be neurotoxic. Treatment with copper chelators reportedly aids in the extraction of Aβ peptides from human AD brain tissue, presumably by disaggregating oligomers, although the extractable Aβ peptide remains only a small fraction of the total Aβ peptide content of the tissue.[222]

Occasional reports suggest that copper accelerates Aβ fibril elongation.[173] It should be noted, however, that assays of fibril elongation based on congophilic dyes such as Congo Red and Thioflavin T may not be quantitatively accurate because their presence can alter the kinetics and nature of the aggregation process.[223] Moreover, it is commonly observed (though not formally reported) that fibrils vary in their affinity for these dyes. If dyes such as Congo Red and Thioflavin T bind to defects in fibril structure (i.e., sites where an Aβ monomer is missing) and if such defects are more prevalent in fibrils that

form rapidly, then accelerations will be overestimated by these measures even when the dye is applied after fibril formation.

The effect of copper:Aβ ratios on aggregation pathways is of interest because different tissue compartments may have widely differing ratios. In general, copper concentrations tend to be much higher than the concentration of Aβ peptides. For example, CSF copper concentrations range from 0.1 to 4.0 μM in various studies,[92,224] while peptide concentrations are orders of magnitude lower, ∼3 nM for Aβ40 and 0.2−0.6 nM for Aβ42.[225] The concentration of the Aβ peptide in extracellular fluid appears to be at least 1 order of magnitude lower than its concentration in CSF,[226] whereas copper concentrations may be as high as 100 μM.[66] Therefore, the Aβ peptide concentration is lower, and the copper:Aβ ratio is higher, in a compartment where amyloid fibrils form, compared to that in CSF where fibrils do not form.

The complex and crowded chemical milieu of the extracellular fluid makes it difficult to ascertain the effective concentration of copper and Aβ peptides, as well as the role of metal ions in nucleating fibril formation in that compartment. It has been suggested that fibril formation may be nucleated when a crowded extracellular or endosomal microenvironment induces the formation of extended β structure in a pair of Aβ peptides, in conjunction with 4-fold coordination of a copper ion by the His13 and His14 residues.[227] By linking two Aβ peptides at position 13/14 via copper coordination, this mechanism would align the peptides for parallel in-register β sheet formation, as found in Aβ fibrils. The copper ion would be released if the β sheet extends to involve residues 13 and 14. An alternative possibility involves dityrosine formation,[228] which would covalently cross-link a pair of Aβ peptides at position 10, similarly aligning the polypeptide chains for parallel in-register β sheet formation. Release of the dityrosine cross-link would not be necessary to form the parallel β sheets found in fibrils.

The thermodynamic stability of Aβ40 fibrils is typically expressed as the monomer concentration in equilibrium with fibrils.[229,230] Reported values for this concentration range from ∼15 μM[231] to <1 μM,[229,232] without regard for the presence of trace metals. A more recent study found the equilibrium monomer concentration to be <100 nM and independent of copper concentration.[187] The differences between these assessments of fibril stability presumably reflect different internal structures and may be significant on several levels. First, the differences underscore the well-documented plasticity of the Aβ peptide structure within a fibril.[186,233−238] Second, they suggest that different laboratories may be studying fibrils with fundamentally different internal structures, thereby accounting for some of the many discrepancies between laboratories. Finally, fibrils with relatively high equilibrium monomer concentrations should not persist in the brain; they should disaggregate into monomers and disperse. Because they do persist in AD, the equilibrium monomer concentration applicable to the fibrils that form in AD must be relatively low.

■ COPPER·Aβ COORDINATION COMPLEXES

Cu(II) most commonly coordinates with four ligands in a square planar geometry. The unpaired electron of Cu(II) makes it possible to infer the identity of these ligands in Aβ·Cu(II) complexes from the superhyperfine structure of electron paramagnetic resonance (EPR) spectra and related techniques. For example, the EPR spectra of Cu(II) complexes with Aβ16, Aβ28, and Aβ40 are essentially identical, indicating that all four ligands are within the first 16 residues of full-length Aβ.[149,155−157,239]

EPR and NMR[240−242] also indicate that several different ligand arrays are present at physiological pH. The situation is simplified below pH 7 where only two distinct arrays are observed, [NH_2^{D1}, O, N_{Im}^{H6}, N_{Im}^{H13}] and [NH_2^{D1}, O, N_{Im}^{H6}, N_{Im}^{H14}].[243,244] These ligand arrays persist above pH 7, but additional arrays are present,[155,156,245] possibly including one in which all three His residues, i.e., [O, N_{Im}^{H6}, N_{Im}^{H13}, N_{Im}^{H14}], are involved.[243,246] The situation again is simplified above pH 9, although the structures that predominate at such high pH may not be present at physiological pH, e.g., those involving deprotonated backbone amide N atoms.[155,247,248]

The identity of the O ligand in these ligand arrays remains unclear. Early investigations suggested that the phenolic O of Tyr10 was involved,[239,249−253] but more recent investigations have concluded that it is not.[145,147,149,155−157,160,243,254] A theoretical analysis points to the involvement of an Asp/Glu side chain ligand,[255] and experimental studies have suggested that the side chain carboxylate of Asp1 is involved[256] either as an equatorial ligand,[157,243] as an axial ligand,[160,257] or through a hydrogen-bonded interaction,[258] in octahedral complexes. Experimental data weigh against the involvement of carboxylate groups from Glu3, Asp7, and Glu11.[258,259] Cross-peaks observed in multinuclear studies with [^{15}N,^{13}C]Ala2 point to the carbonyl oxygen of Ala2 as the O ligand (Figure 2c),[259] but the low amplitude of these cross-peaks leaves open the possibility that the Ala2 carbonyl oxygen may not be the O ligand in all ligand arrays.[254] The involvement of the Ala2 backbone carbonyl group may lead to the elimination of residues Asp1 and Ala2, and the conversion of Glu3 into pyroglutamate.[259,260] N-Terminally truncated forms of Aβ are common in the amyloid plaques of AD, including those in which residue 3 is an N-terminal pyroglutamate.[261−268]

X-ray absorption spectroscopy (XAS) and related techniques have suggested somewhat different conclusions about the coordination state of copper in copper·Aβ complexes.[269] An XAS study of the Aβ16·Cu(II) complex has been interpreted as indicating a square planar ligand array with two His residues,[270] while studies of the Aβ40·Cu(II) complex point to a pentacoordinated complex involving three His residues, Tyr, and water.[253,271] Other investigators have collected similar spectra but interpreted their results to indicate that six ligands were involved: three His residues and a carboxylate in equatorial positions with water and another carboxylate in axial positions.[160] Raman spectroscopy of amyloid plaques from human AD brain also indicates the presence of copper−His coordination[145] and suggests that copper also binds to deprotonated backbone peptide groups at pH 7.[250]

The prevailing opinion seems to be that Cu(II) forms various square planar coordination complexes with three N ligands and one O ligand among the first 14 residues of Aβ peptides. The identity of these ligands is less clear than the evidence that several different coordination modes are present at physiological pH. The lack of consensus about which Aβ ligands are involved in copper binding may be due to differences in buffer conditions, pH, and preparation methods. The practice of cooling samples to ≤77 K for EPR and XAS studies may also cause changes in the structure or distribution of structures compared to those under physiological conditions. It is unclear whether aggregation state affects the structure of complexes, although such effects would be expected, and the existence of a neurotoxic dimer formed by a His−Cu(II)−His bridge has

been suggested.[272] Nevertheless, the EPR spectra of Cu(II) complexes with monomeric, oligomeric, and fibrillar Aβ peptides are similar in appearance.[148,273] The morphology of Aβ fibrils as revealed by electron microscopy is also reportedly unaffected by the addition of Cu(II);[273] however, the images were of low resolution, and the possible confounding effects of negative staining with a high concentration of heavy cations were not considered.[187]

Compared to Cu(II), very little information is available on the structure of Cu(I) complexes with Aβ peptides. Cu(I) most commonly coordinates with four ligands in a tetrahedral geometry, but an early computational study suggested that Cu(I) would most likely form a tricoordinated complex with two imidazole rings and a carbonyl oxygen.[274] Nevertheless, Cu(I) complexes with Aβ16 and Aβ40 are not very reactive with O_2, which is more consistent with a linear two-coordination model than with a three-coordination model.[270] XAS studies of Cu(I)·Aβ complexes have been interpreted as suggesting a linear complex with two imidazole ligands,[270] which is consistent with mass spectrometry evidence that both His13 and His14 are involved in the binding of Cu(I) to Aβ16.[179]

Understanding the structure of copper·Aβ complexes under various conditions is relevant to an understanding of AD pathogenesis because their structure has a profound effect on the redox behavior of the copper ion, the tendency of Aβ peptides to aggregate and form fibrils, and their interactions with other cell components. For example, copper binding at pH 5.5−6.5 induces Aβ40 to penetrate lipid bilayers,[239,251] suggesting that when copper·Aβ complexes are endocytosed and acidified they may take on a ligand array that forms and becomes disruptive to lipid membranes only at low pH. Another example is that copper binds equivalently to Aβ42 synthesized either from all D-amino acids or from all L-amino acids, but only the all-L form binds to phosphatidylserine in lipid membranes and exhibits neurotoxicity.[275] Of course, conclusions based to any extent on the relative neurotoxicity of copper·Aβ complexes must be approached cautiously because it is not clear which measures of neurotoxicity are relevant to the pathogenesis of AD, and there are many instances of apparent contradiction. For example, studies of Aβ peptide uptake and intracellular thiol levels suggest that the formation of copper·Aβ complexes may be neuroprotective.[276]

COPPER AND THE MEASUREMENT OF OXIDATIVE STRESS

Oxidative stress in a biological system is typically defined by its chemical mechanisms and consequences. The mechanisms have in common the removal of an electron (or an increase in the formal oxidation state) from a reference compound by an oxidizing agent, while the consequences are usually the creation of new compounds that would not otherwise be created via enzymatically controlled biochemical pathways. There is widespread acknowledgment that oxidative stress is a prominent feature of AD,[277−284] although it is not yet established whether oxidative stress is a cause or consequence of the underlying pathological process.

Cells and tissues are replete with mechanisms that protect against oxidative stress, usually by the sacrifice of an electron from a "decoy" compound that may be regenerated later, e.g., glutathione. Consequently, cells and tissues tend to be reducing environments. Cu(II) in this type of environment is problematic because its reduction yields a potent and promiscuous reducing agent, namely Cu(I), which has the ability to create various highly reactive oxygen species from molecular oxygen. It has been suggested that the accumulation of Aβ peptides in the brain may be a protective response to oxidative stress, by virtue of their ability to bind and sequester copper ions in both of its oxidation states.[285−292] However, a somewhat larger body of evidence suggests that Aβ peptides promote oxidative stress through redox cycling of copper while bound to an Aβ peptide.[251,289,293−296]

Part of the difficulty in resolving issues about the relationship among copper, Aβ peptides, and oxidative stress is that oxidative stress is inherently difficult to quantify. Innumerable approaches have been devised to do this, but each approach has limitations and inherent problems. The approaches that have been applied in AD fall into four main groups. The most commonly employed approach is to assay specific toxic products such as acrolein,[297−300] malondialdehyde,[78,80,301,302] hydroxynonenal,[283,296,303−306] and 7β-hydroxycholesterol.[307,308] One problem with this approach is that each analyte is the product of oxidative lipid degradation. Hence, these assays report only oxidative damage to lipids. Moreover, they are not produced stoichiometrically during oxidative stress because of free radical chain reactions and independent redox cycling, so they are only semiquantitative measures of oxidative stress. They also tend to be chemically reactive so that the amount of these substances available for assay is only the net difference between the amount made and the amount that has already reacted with something else. Finally, when they are measured as thiobarbituric acid reactive substances (TBARS, often assumed to be primarily malondialdehyde),[281,309−314] there are multiple other substances that interfere with the assay.[315]

A second approach to quantifying oxidative stress in AD is to assay relatively stable products of oxidative stress such as isoprostanes, neuroprostanes, and neurofurans.[316−323] Once again, these assays measure only products of lipid damage, and they are not produced in stoichiometric quantities. The assays also require sophisticated mass spectrometry instrumentation and isotopically labeled internal standards. Being relatively nonreactive, however, they are eliminated without being metabolized, and the amount measured in an assay of daily urinary output tends to reflect more accurately the rate of production.

A third approach is to assay the concentration of various antioxidants such as glutathione,[324−327] ascorbate,[328,329] and α-tocopherol.[327,330] Alternatively, the antioxidant activity in a tissue or fluid may be measured by challenging the material with an oxidant and measuring either the degree to which a free radical reaction is quenched[85] or the time lag before oxidation products are produced.[291,331] An obvious problem with the former approach is that one cannot assay all possible antioxidants, while the latter approach yields results that vary with the nature of the oxidative challenge.

A fourth approach is to assay for nucleic acids[332−335] or proteins that have been chemically altered, e.g., by nitration[336,337] or carbonylation.[338−346] Carbonyl groups may form through the direct addition of an oxygen atom, typically to a His or Met residue.[347] However, a detailed mechanistic study of protein carbonylation concluded that new carbonyl groups on proteins are more likely to arise from the attachment of lipid-derived products of oxidative stress than from direct oxidation of amino acid side chains.[348]

The modification of proteins by oxidative lipid degradation products and "membrane-associated oxidative stress"[349] is a recurring theme in the literature of Alzheimer's disease pathogenesis. For example, copper·Aβ complexes accelerate the production of hydroxynonenal from arachidonate which, in turn, covalently reacts with the His residues on Aβ.[350] The modification of His by hydroxynonenal yields a hemiacetal product with a lone pair of electrons on the imidazole ring that can still coordinate with copper ions.[351] Thus, hydroxynonenal modification increases the membrane affinity of an Aβ peptide, and if it carries a bound copper ion, it can concentrate redox-active copper at lipid membranes.[305] In vitro, HNE modification not only increases further lipid peroxidation but also accelerates amyloid fibril formation.[295] "Membrane-mediated amplification of amyloidogenesis" is observed even within the complex chemical milieu of a human brain lipid extract, which is noteworthy because it provides some assurance that such phenomena are not artifacts of a chemically refined environment.[296]

Aβ peptides that have been modified by lipid oxidation products such as hydroxynonenal are difficult to sequence with ordinary electrospray ionization mass spectrometry.[352,353] This type of analysis typically relies on the digestion of Aβ peptides by trypsin, which yields y-cations bearing C-terminal Lys and Arg residues for sequencing. With Aβ peptides, digestion by AspN has been more successful.[296] This treatment yields Aβ(1−6), Aβ(7−22), and Aβ(23−40/2) segments with N-terminal Asp residues and b-cations that are readily sequenced.

Another type of chemical alteration observed in AD is the formation of dityrosine, which is found in relatively high concentrations in regions of brain that tend to be affected by AD.[228] In vitro studies have demonstrated that copper ion binding promotes dityrosine formation,[354] but only when copper:Aβ ratios are at least 1:1.[191] Therefore, it has been suggested that dityrosine formation follows rather than drives aggregation and fibril formation.[355] It has also been reported that peroxidase activity specifically induces dityrosine formation.[356,357]

At one point, it was suggested that Aβ peptides split into fragments that are both neurotoxic and able to generate additional oxygen radicals.[358,359] However, these findings have since been strongly refuted.[360] Instead, Aβ peptides may be particularly effective at quenching reactive oxygen radical species by undergoing oxidative damage themselves, a trait that they may share with the prion protein, PrP^C.[361,362]

COPPER AND THE PATHOGENESIS OF OXIDATIVE STRESS

There are diverse perspectives on the relationship between copper·Aβ complexes and neurotoxicity. Some regard copper··Aβ complexes as direct neurotoxins.[251,272,363,364] Others find that Aβ peptides attenuate the toxicity that copper seems to otherwise exert by itself,[152,189,285,365] perhaps through an interaction with copper-binding cell-surface proteins such as APP,[366,367] or by eliminating a membrane disrupting activity exhibited by copper.[18,368] Many have suggested that the neurotoxicity of Aβ peptides is due to oxidative stress, associated with the redox cycling of copper ions bound to Aβ peptides and the production of hydrogen peroxide.[189,310,329,364,369,370]

When considering how copper·Aβ complexes produce hydrogen peroxide, uncertainty in the reduction potential of the complex is a problem. Cyclic voltammetry studies have estimated it to range from a high of 770 mV[364] to lower values such as 340 mV[371] or 280 mV[159] (all potentials mentioned are referenced to the standard hydrogen electrode).[159,372−375] Jiang et al. have suggested that the most reasonable value may be as low as 100 mV.[159] This uncertainty may be due to various factors, including the use of indirect detection methods for Cu(II) reduction, the use of redox-active buffers, alternative conformations for the peptide,[376] the proximity of a redox-active thioether group,[377] and variations in potential due to concentration effects,[378] especially the intracellular concentrations of various oxygen species.[274] The use of reporters such as tris(2-carboxyethyl)phosphine (TCEP) or 2′-7′-dichlorofluorescein (DCFH) for measuring H_2O_2 production[161,379] may yield misleading results by redox coupling to the reaction being measured, and by redox cycling in a manner that is stoichiometrically independent from the reaction of interest. On top of these uncertainties, the likelihood of a redox reaction in vivo may differ from that predicted by simple comparison of standard reduction potentials for the half-reactions involved because they are not occurring under "standard" conditions. The likelihood and direction of the reaction will be affected by the concentration of reactants, the occurrence of other coupled reactions, the temperature, and the chemical environment of the ionizing species.

There are several noteworthy aspects to the redox activity of copper·Aβ complexes. First, binding to Aβ does not by itself tend to facilitate the reduction of Cu(II).[155,160,161,251,364] Even the highest reported reduction potentials for the copper·Aβ complex are all smaller than those of the most vulnerable amino acid side chains such as Met (1.5 V)[380] and Tyr (930 mV[381] or 960 mV[382,383] at pH 7.4), so one would not expect Cu(II) reduction merely upon formation of the complex. However, Cu(II) may be reduced prior to complex formation by peptide bonds (even peptide bonds within the Aβ peptide) via the well-known Biuret reaction, which forms the basis for many protein concentration assays. The Met35 side chain is oxidized under some conditions,[347,362,384,385] in spite of the much larger reduction potential for this thioether than for Cu(II), which supposedly drives the reaction.[159] The vulnerability of Met35 appears to be explained by the involvement of a suitably positioned backbone amide group, which can lower the reduction potential of thioethers and thereby promote sulfur oxidation.[386−388] In other studies, His13 and His14 are the targets of oxidative damage, forming 2-oxo-His[347] with relative sparing of His6.[389,390]

Second, it has been suggested that Aβ-bound Cu(II) may be reduced in the course of oxidizing compounds such as cholesterol, L-DOPA, dopamine, ascorbate, borohydride, pyruvate, and glutathione.[372−375] However, some have provided experimental data indicating that Aβ·Cu(II) complexes are not reduced by cholesterol or dopamine.[161] In any case, it is clear that Aβ·Cu(II) complexes can promote the oxidative degradation of polyunsaturated fatty acyl (PUFA) chains such as arachidonate, for which a reduction potential of 0.6 V has been reported.[391] The mechanism requires dissolved O_2, an electron donor such as ascorbate, and the participation of His13/14 and Met35, but with no net oxidative damage to those residues.[295,392] The oxidative degradation of arachidonate typically yields products such as hydroxynonenal, which are of considerable interest because their concentrations are increased in the cerebrospinal fluid of persons with AD.[280,393−395]

Third, PUFA oxidation products such as hydroxynonenal are characteristic of reactions initiated by hydroxyl radicals. A well-

known scenario for the production of hydroxyl radicals in brain tissue begins with the donation of electrons by ascorbate. The concentrations of ascorbate in the CSF are typically 150 μM,[396] and levels in the extracellular fluid may be as high as 400 μM.[397] Ascorbate reduces the Aβ·Cu(II) complex to the Aβ·Cu(I) complex, which in turn reduces molecular oxygen according to the reaction $2A\beta\cdot Cu(I) + O_2 + 2H^+ \leftrightarrow 2A\beta\cdot Cu(II) + H_2O_2$.[398] This reaction implies that the Aβ·Cu(II) complex has a reduction potential on the low end of the values reported above because higher values would drive this reaction to consume hydrogen peroxide, not produce it.[159] The hydrogen peroxide produced may react with other Cu(I) ions to produce hydroxyl radicals via the copper analogue of the Fenton reaction: $Cu(I) + H_2O_2 \leftrightarrow Cu(II) + OH^- + {}^{\bullet}OH$. Cu(I) appears to be prevalent in the brain, especially in the hippocampus and midbrain,[100] although its physical state is unknown. The hydroxyl radicals produced are even more potent mediators of oxidative stress than hydrogen peroxide,[378] and most likely responsible for PUFA oxidation as well as in vitro dityrosine formation in Aβ that is treated with copper and ascorbate.[399]

Fourth, iron may also redox cycle upon binding to Aβ peptides[400−402] and create free radicals.[403] Zinc cannot redox cycle, but it may have other effects such as inhibiting peroxide production, modulating various measures of neurotoxicity,[189,195,293,404−406] and causing the accumulation of toxic Aβ oligomers when released at synapses with neurotransmitters.[407]

Fifth, the source of oxygen for peroxide production is dissolved molecular dioxygen, which may be trapped in some way by the reduced copper·Aβ complex.[310] The reduction of dioxygen to peroxide requires two electrons, and it normally proceeds through a superoxide radical anion intermediate. However, a superoxide intermediate has not been detected.[364] Instead, calculations have suggested that external reducing agents such as ascorbate or glutathione may initiate the reaction by donating a single electron to Cu(II). Dioxygen then binds to Cu(I), which donates an electron to regenerate Cu(II) and produce superoxide. Finally, another external reducing agent donates an electron directly to superoxide via proton-coupled electron transport while it remains bound to the copper, thus bypassing the free superoxide radical step.[408]

Sixth, the redox activity of copper·Aβ complexes appears to require the participation of a thioether, such as Met35. Redox activity does not occur with copper·Aβ28 complexes (which lacks Met35) or with Aβ peptide variants in which Met35 is substituted.[159,296,364,371,385,409] Mutations in the vicinity of Met35 such as I31P also abolish these reactions, presumably through an effect on local structure,[410] but homocysteine[214] and methionine[251] added apart from the Aβ peptide can promote them. Because the Met35 side chain is not consumed,[411] it somehow facilitates these reactions in a way that was at one time proposed for Tyr10[412] but cannot be explained with standard reduction potentials.

Finally, metallothionein-3 (MT-3) may be have a role in sequestering free Cu(II) ions. With a full complement of seven Zn(II) ions (i.e., Zn_7-MT-3), the zinc−thiolate clusters can exchange Zn(II) for Cu(II).[405] Four Cu(II) ions are reduced to Cu(I) upon binding, in conjunction with the formation of two disulfides; however, the Cu(I) ions are redox-inactive, and hydroxyl radical production is quenched. Zn_7-MT-3 may swap Zn(II) for Cu(II) in copper·Aβ complexes and thereby detoxify them.[406] In this process, there appears to be no specific interaction between Zn_7-MT-3 and the copper·Aβ complex.[413]

COPPER AND THERAPEUTIC INTERVENTION IN AD

The rationale for treating AD with copper chelators is based on several of the observations described above, including the elevated peripheral blood levels of copper in AD, the accumulation of copper in amyloid plaques in human disease, the aggravation of AD-like pathology in animal models on dietary copper supplements, and the ability of copper chelators to disaggregate and help extract Aβ in tissues. In addition, a clinical trial of the copper chelator D-penicillamine in human AD patients found that biomarkers of oxidative stress were reduced.[414]

However, D-penicillamine is not specific for copper and does not cross the blood−brain barrier. Therefore, early trials were conducted with clioquinol (CQ, 5-chloro-7-iodo-8-hydroxyquinoline) because it can cross the brain−blood barrier and has sufficient affinity for Cu(II) and Zn(II) to compete with Aβ peptides for these ions.[415−417] Perhaps because of its copper chelating ability, CQ suppressed the toxicity of Aβ42 and Aβ42 with and without an oxidized Met35 residue in primary neuronal cultures.[418]

In addition to copper chelating ability, CQ activates signaling pathways that upregulate matrix metalloproteinases in cultured CHO cells that in turn degrade Aβ peptides and decrease their level of secretion.[419] This property is shared among several lipophilic metal-binding compounds that elevate intracellular copper levels,[135,146,420] and synthetic nanocarriers have been developed with similar effects.[421] The activity of proteinases against Aβ peptides may be enhanced when compounds such as CQ remove copper from aggregated proteins.[422] CQ may also alter the nature of Aβ peptide−lipid interactions,[423] alter the tendency of oligomeric Aβ forms to accumulate at active synapses,[407] and suppress caspase-3 activation by pro-apoptotic agents.[424]

In mouse models, CQ dramatically reduces the level of deposition of Aβ peptide in brain tissue,[415,425,426] while radioiodinated CQ concentrations were increased in a mouse model of AD and the brains of humans with AD.[427] A phase 2 human clinical trial of CQ in persons with moderately severe AD suggested that it slowed cognitive decline and reduced the plasma level of the Aβ peptide.[428] Despite the absence of demonstrated toxicity in recent human trials, concerns about the neurotoxicity of CQ persist because of clear toxicity at high doses in animals.[429,430]

An 8-hydroxyquinoline derivative of clioquinol known as "PBT2" with advantageous synthetic and pharmacokinetic features has been tested in animal models[431] and introduced into human trials. In a placebo-controlled trial, PBT2 caused a significant decrease in CSF Aβ42 levels but no change in serum Aβ42 levels, or in serum and CSF concentrations of Aβ40, tau, copper, and zinc.[432,433] Nevertheless, there was an improvement in some measures of cognitive performance.

Oral zinc therapy has been suggested for the treatment of AD,[434,435] based on its ability to reduce copper uptake in the intestine,[40] an approach that has some utility in the treatment of Wilson's disease. Another copper-based therapeutic strategy involves the use of targeted artificial proteases.[436,437] Using the KLVFF sequence as a targeting motif, a cyclen derivative is able to remove copper from amyloid deposits, become proteolytically active, and cleave the Aβ into nontoxic fragments. Cyclen and cyclam by themselves may chelate copper, reduce oxidative stress, and reduce the toxicity of copper·Aβ complexes.[438] Naturally occurring compounds such as curcumin may also

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share this activity.[439] Finally, synthetic copper ligands can deliver copper to intracellular pools and inhibit GSK-3 via akt signaling in cell culture,[136] with promising results in mice.[135] However, one compound has been shown to bind Aβ42 as well as a copper ion and result in increased toxicity.[440]

CONCLUSIONS AND FUTURE RESEARCH PRIORITIES

Understanding the transcellular circulation of copper and its physiological role in neurons must be a top priority for future research on AD. APP is only one component of a complex system that regulates copper levels in various tissue compartments by transporting ions across membranes, along axons, and out into synaptic clefts via secretory vesicles, yet the role of this copper transport activity in health or disease is not known. The effects of APP mutations that cause familial AD should be explored for their effects on copper transport apart from their effects on Aβ peptide production. Understanding copper circulation will provide important insight into the circumstances under which copper·Aβ complexes form and into the mechanism by which drugs that alter intracellular copper levels are linked to Aβ degradation by metalloproteinases or tau hyperphosphorylation by GSK-3.

Another high priority must be understanding the reasons that Aβ peptides, copper·Aβ complexes, and amyloid fibrils exhibit different properties in different laboratories. Peptides, complexes, and fibrils exhibit a remarkable degree of polymorphism, and amyloid plaques in AD are clearly heterogeneous in composition. However, it is not clear which in vitro preparations accurately mimic the properties of significant components in amyloid plaques, leaving us at risk of drawing misleading and unhelpful conclusions from them.

Finally, the field needs a better approach to neurotoxicity assessment. Many potentially neurotoxic mechanisms have been suggested, many of which involve copper implicitly, if not explicitly.[441] However, a central problem with current approaches to assessing neurotoxicity in AD research is that the time scale of laboratory toxicity assessments (minutes to hours) is much shorter than the time scale in animal models (days to months), which, in turn, is much shorter than the time scale in actual AD (presumably years). There is no means at present to test whether any given in vitro or in vivo assessment of neurotoxicity is a valid surrogate measure for the neurotoxicity mechanisms that operate in AD.

AUTHOR INFORMATION

Corresponding Author

*E-mail: axe@pharm.med.upenn.edu. Phone: (215) 898-5000.

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ABBREVIATIONS

Aβn, n-residue peptide beginning with APP residue Asp672; A$\beta(n-m)$, peptide beginning with APP residue n + 671 and extending to residue m + 671.

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