



## Bioinorganic Chemistry of Alzheimer's Disease

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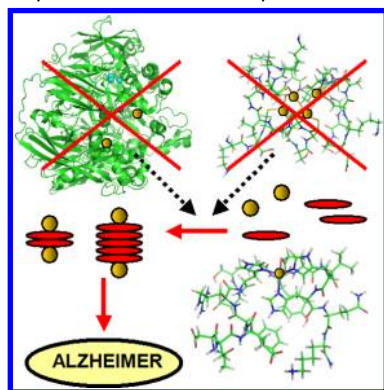
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## Bioinorganic Chemistry of Alzheimer's Disease

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### 1. INTRODUCTION: ALZHEIMER'S DISEASE FROM A CHEMIST'S POINT OF VIEW

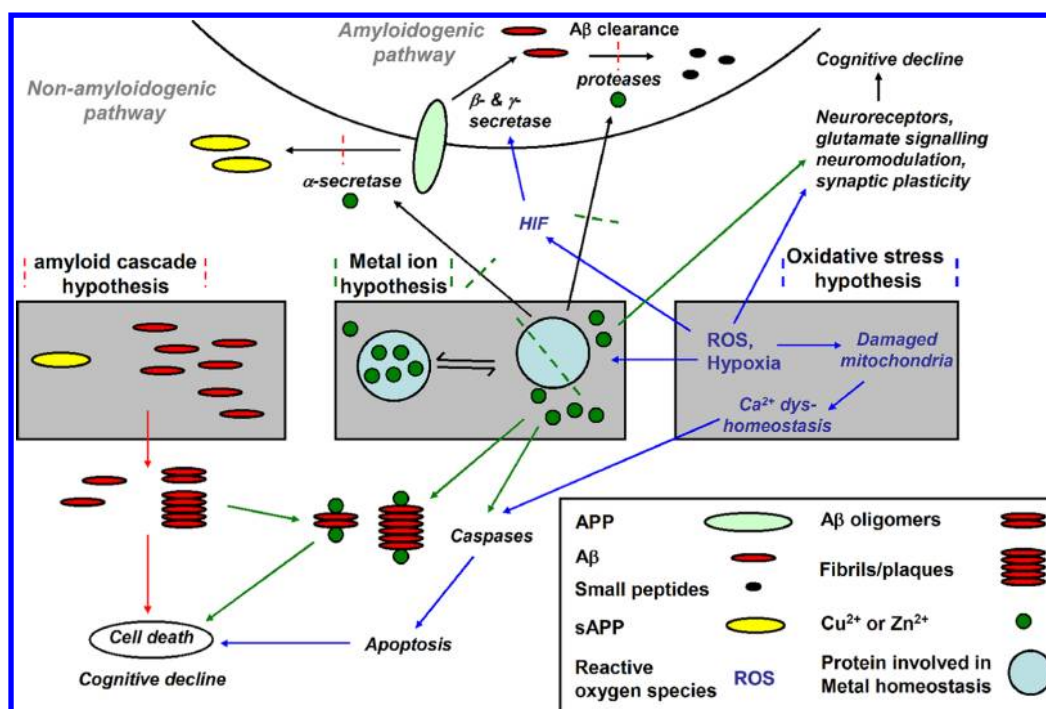
#### 1.1. Definitions and Symptoms

Alzheimer's disease (AD)<sup>1–3</sup> is the most common form of dementia (estimated ~50–60% of all cases), associated with loss of memory (in particular episodic memory), cognitive decline, and behavioral and physical disability, ultimately leading to death.<sup>4–6</sup> It is the sixth most common cause of death in the US according to the Alzheimer's Association, and more than 5 million Americans suffered from the disease in 2011, with prevalence growing steadily.<sup>7</sup> A large body of recent research, to be reviewed herein, has put the AD field into contact with bioinorganic chemistry, and this review will attempt to present the growing role of bioinorganic chemistry in AD research, with a particular emphasis on zinc homeostasis.

The two main histopathological criteria for AD are observations of extracellular deposits of fibrillar peptides called senile plaques and of widespread intraneuronal fibrillar tangles.

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**Figure 1.** Simple overview of three hypotheses of Alzheimer's disease: (i) amyloid cascade hypothesis, with  $A\beta$  accumulation (red) being a main pathogenic event; (ii) metal ion hypothesis, with metal ion (green) dyshomeostasis leading to amyloid imbalance; and (iii) oxidative stress hypothesis, with oxidative and general stress (blue) leading to mitochondrial damage, metal ion dyshomeostasis, apoptosis, and  $A\beta$  imbalance.

The senile plaques are formed from ~40-residue fragments, known as  $\beta$ -amyloids ( $A\beta$ ), of the transmembrane amyloid precursor protein (APP) found in the membranes of cells and organelles such as mitochondria;<sup>4,5</sup> thus, recent reports show that  $A\beta$  also accumulates intracellularly.<sup>8</sup> The neurofibrillar tangles consist of twisted strands of hyperphosphorylated tau protein, a protein that is important for the structural integrity of microtubules, structural tubulin polymers of the cytoskeleton of the neurons,<sup>4</sup> and are thus commonly observed upon neurodegeneration. Amyloid plaques are also not unique to AD, because 20–40% of unaffected elderly individuals possess amyloid plaques sufficient for post-mortem AD diagnosis,<sup>9</sup> showing that the disease requires a clarifying and unifying pathogenic understanding.

Other observations consistently relating to AD brains are (1) loss of neurons and synapses in the cerebral cortex, involved in memory and cognition, and in particular in the hippocampus (part of the limbic system), playing a key role in memory,<sup>10</sup> (2) oxidized biomolecules reminiscent of oxidative stress,<sup>11,12</sup> (3) impaired energy metabolism and reduced glucose uptake,<sup>13</sup> (4) altered calcium,<sup>5</sup> iron,<sup>14,15</sup> zinc,<sup>16</sup> and copper<sup>17</sup> homeostasis,<sup>18,19</sup> (5) diabetes-like pathologies,<sup>20,21</sup> and (6) elevated homocysteine<sup>22–24</sup> and abnormal expression of homeostatic metalloproteins such as metallothioneins.<sup>25</sup> As will be discussed, all of these pathological changes have relations to metal ion homeostasis that may point toward underlying common causes of AD.

Cognitive and behavioral symptoms associated with AD<sup>6,26</sup> include (1) mild cognitive impairment (MCI) before actual AD diagnosis,<sup>4,27</sup> (2) progressive impairment of activities of daily living, (3) enhanced risk of depression,<sup>28,29</sup> (4) memory loss, in particular loss of episodic memory, that is, memory associated with own life experiences, distinguishing AD from other types of dementia,<sup>26</sup> (5) loss of identity,<sup>30</sup> and (6) ending with severe and global impairment of cognition and mobility.<sup>10,26</sup>

## 1.2. Risk Factors

AD is a complex disease, mostly occurring sporadically with no apparent inheritance<sup>31</sup> and with age as the main risk factor.<sup>4</sup> The perceived complexity of AD implies that the disease has a broad clinical spectrum<sup>6</sup> aggravated by a multitude of genetic and environmental factors and is affected by many genes of small but cumulative significance.<sup>4,32,33</sup> Exogenous risk factors include (1) brain trauma,<sup>7</sup> (2) smoking,<sup>29,34</sup> (3) obesity,<sup>35,36</sup> (4) diabetes,<sup>4,37</sup> (5) hypertension,<sup>29,38</sup> (6) cholesterol,<sup>39,40</sup> (7) elevated homocysteine levels (hyperhomocysteinemia),<sup>22,24</sup> and (8) exogenous metal exposure,<sup>41</sup> including lead,<sup>42</sup> mercury,<sup>43</sup> and aluminum.<sup>44–47</sup>

Genetic risk factors are now known to cause AD in rare cases, most notably due to mutations in APP or presenilin,<sup>48</sup> which is a constituent of the  $\gamma$ -secretase enzyme complex that degrades APP into  $A\beta$  giving rise to familial AD,<sup>5</sup> and due to isoforms of the cholesterol transporter apolipoprotein (ApoE), ApoE4, which increase risk of sporadic AD by 15 times when they are homozygotic.<sup>5</sup> Other genes, for example, coding for clusterin and phosphatidylinositol-binding clathrin assembly protein,<sup>49,50</sup> have recently been implicated by genome-wide association studies<sup>51</sup> and explain about half of the genetic background of sporadic AD.<sup>52</sup> The mechanism of their involvement is not completely understood although they seem to be implicated in cholesterol metabolism, immune defense, or synaptic function.<sup>52</sup>

Because the pathogenic mechanism of AD is currently not understood, current treatments are mainly symptom-relieving and are typically effective for up to a year at best.<sup>18,53</sup> Among such treatments, focus has mainly been on relieving cognitive symptoms, for example, *N*-methyl-D-aspartate (NMDA) receptor antagonists (memantine) or acetylcholinesterase inhibitors (donepezil).<sup>4,54</sup> Given the poor therapeutic effects of current treatments, prevention has instead been suggested to

provide better protection against AD.<sup>55,56</sup> However, recent developments at the interface between bioinorganic chemistry and neuroscience have opened the door for appealing new targets relating to zinc homeostasis, to be discussed in this review.

Cumulated evidence shows that the risk of AD can be reduced both by avoiding the previously mentioned exogenous risk factors, including enhancing neuronal capacity, for example, by education,<sup>57,58</sup> and intake of vitamin B<sub>12</sub>, folate, antioxidants such as vitamin E (evidence for vitamin C is not conclusive),<sup>55</sup> unsaturated fatty acids,<sup>55,59</sup> cereal, and fish, or controlled caloric restriction,<sup>60</sup> and regular mental<sup>61</sup> and physical activity.<sup>56,57</sup> Some factors may correlate with but not cause disease progression: For example, education is a key component of an active cognitive life style that may reduce risk of AD,<sup>57,58</sup> but education may correlate with other factors such as dietary intake. Distinguishing correlation and causation is thus central also to AD research.

## 2. AD PATHOGENESIS: THREE CURRENT COMPETING HYPOTHESES

Since the days of the cholinergic hypothesis of AD,<sup>62,63</sup> acetylcholinesterase inhibitors such as donepezil have dominated the market for AD treatment, although this paradigm only delays cognitive decline by typically one year.<sup>53,54</sup> The limited efficiency of these symptom-treating receptor antagonists has led to quests for more causative pathogenic targets.<sup>10,64</sup> During the past decade,<sup>4,10,16,264</sup> three main hypotheses on the pathogenesis of AD have emerged that focus on different features of the disease and are to some extent seen as competing:<sup>10</sup> the amyloid cascade hypothesis,<sup>4,5,65</sup> the metal ion hypothesis,<sup>17,66–69</sup> and the oxidative stress hypothesis.<sup>70–72</sup> A crude overview of some central ideas of these three hypotheses is given in Figure 1.

The amyloid cascade hypothesis states that impaired balance between A $\beta$  production and clearance is the main cause of AD and that amyloids are the main neurotoxic substances in AD.<sup>5</sup> Consequently, this hypothesis favors treatments that inhibit A $\beta$  production or enhance A $\beta$  clearance in the AD brain.<sup>4</sup>

The metal ion hypothesis states that the underlying cause of AD is impaired metal homeostasis, in particular of Zn, Cu, and Fe, with A $\beta$  imbalance being a consequence of this.<sup>67,68</sup> This hypothesis favors treatments such as chelators that address the metal ion imbalances supposedly causing amyloid accumulation.<sup>73</sup>

The oxidative stress hypothesis asserts that age-enhanced or genetically and environmentally enhanced oxidative stress results in accumulated gene defects and declining mitochondrial function that subsequently leads to neurological disorders, either gradually or when reaching a critical threshold that initiates apoptosis in neurons.<sup>70,74,75</sup> Apoptosis occurs in a wide range of neurological disorders and by a range of pathways that can be triggered, for example, by lesions, misfolded proteins, oxidative stress, excitotoxicity, or Ca<sup>2+</sup> dyshomeostasis.<sup>76</sup>

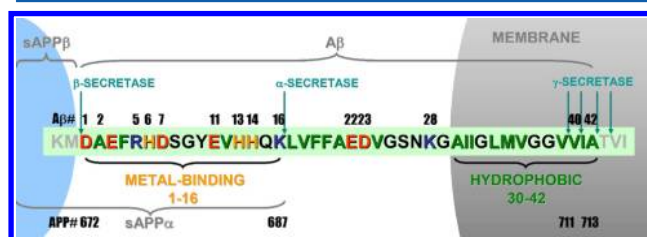
## 3. AMYLOID CASCADE HYPOTHESIS

### 3.1. Production of A $\beta$

The amyloid cascade hypothesis<sup>65</sup> focuses on the hallmark symptom of the disease, the senile plaques, asserts that amyloid imbalances are the main, pathogenic cause of AD, and was primarily supported by genetic risk factors of rare familial AD affecting the production or clearance of A $\beta$ .<sup>4,77,78</sup> Mechanisms

of A $\beta$  formation and clearance have been discussed in recent reviews,<sup>8,79</sup> and many chemical and structural aspects of amyloids have been discussed in the review by Rauk.<sup>80</sup>

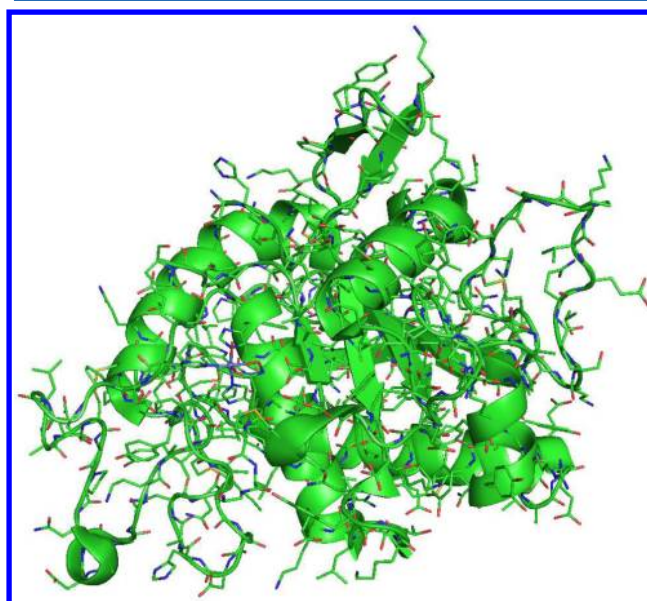
A $\beta$  is derived from cleavage of the membrane protein APP found both in the outer cell membrane, accessible from the extracellular environment, and intracellularly, in membranes of organelles such as mitochondria.<sup>8</sup> The A $\beta$  sequence within APP is shown in Figure 2. There are two main pathways of APP



**Figure 2.** The A $\beta$  sequence within APP and the main secretase cleavage positions. Both A $\beta$  numbering (A $\beta$ #) and APP numbering (APP#) is indicated. Amino acids are colored according to negative charge (red), positive charge (blue), histidines (orange), and hydrophobic (green).

cleavage, the amyloidogenic and the nonamyloidogenic; both of them occur in the normal, healthy organism, and A $\beta$  exists in all living persons and serves roles in the healthy brain.<sup>81</sup>

The nonamyloidogenic cleavage of APP at the  $\alpha$ -cleavage site, which lies within the region of formation of A $\beta$  and therefore precludes its later formation, is catalyzed by a group of proteases called  $\alpha$ -secretases and leads to formation of innocent sAPP $\alpha$  peptides outside the cell.<sup>8,82,83</sup> The  $\alpha$ -secretases belong to the ADAM family (a disintegrin and metalloprotease family), for example, ADAM9, ADAM10, and ADAM17,<sup>8</sup> and are membrane zinc proteases.<sup>84</sup> As an example, a structure of ADAM17 (TACE) with a bound inhibitor is shown in Figure 3.<sup>85</sup> In contrast, an aspartyl protease cleaves APP at the  $\beta$ -site and is referred to as  $\beta$ -secretase (beta-site



**Figure 3.** The zinc protease ADAM17 (TACE) with  $\alpha$ -secretase activity (3L0T.pdb). Active-site Zn(II) (blue) coordinates three histidines and two oxygen atoms from an inhibitor. See ref 85. Picture made with Pymol.



APP cleaving enzyme 1, BACE1).<sup>86,87</sup> Subsequent cleavage by the enzyme complex  $\gamma$ -secretase leads to formation of the two variants A $\beta$ 40 (ca. 90%) and A $\beta$ 42 (ca. 10%), with 40 and 42 residues, respectively.<sup>8,10</sup>

### 3.2. A $\beta$ Production–Clearance Imbalances in AD

Any disruption that affects the balance between the two pathways can alter the balance between innocent cleavage products and A $\beta$ : For example, low pH ( $\beta$ -secretase has a low  $\text{pH}_{\text{opt}}$ ),<sup>8</sup> oxidative stress or hypoxia (which induces  $\beta$ -secretase<sup>88–90</sup>), hypercholesterolemia ( $\beta$ -secretase and  $\gamma$ -secretase are inhibited by reduced cholesterol levels),<sup>91</sup> and lack of Zn(II) in the  $\alpha$ -secretase active sites may be hypothesized to affect A $\beta$  production. Accumulation of homocysteine known to occur in AD<sup>24</sup> enhances expression of  $\gamma$ -secretase in the rat brain,<sup>92</sup> also shifting the balance toward more A $\beta$ .

Also, any disruption of the clearance of A $\beta$  will lead to amyloid accumulation, which is also of central importance.<sup>93</sup> To maintain a normal healthy concentration of A $\beta$  in the brain, A $\beta$  is rapidly degraded by a number of proteases, including neprilysin, insulin-degrading enzyme, angiotensin-converting enzyme, and matrix metalloprotease-2 and -9.<sup>94,95</sup> Thus, in the healthy brain, the production by secretases and the clearance of A $\beta$  by other proteases is in balance (typically at rates of ~8% per hour), so that amyloids do not accumulate.<sup>96</sup> Neprilysin is considered the main A $\beta$  protease<sup>97,98</sup> that degrades both monomers and oligomers of A $\beta$ .<sup>99</sup> To be discussed in this review, it is notable that both nonamyloidogenic and A $\beta$ -clearing proteases depend on bound zinc in active sites, whereas free Zn<sup>2+</sup> ions contribute to the amyloidogenic pathway and also stabilize A $\beta$  against degradation.

### 3.3. Structural Forms and Toxicity of Amyloids

The two A $\beta$  peptides can be found in various forms once produced: The simple peptides react to produce (i) soluble oligomers, (ii) protofibrils, and (iii) extracellular aggregates or fibrils, which are insoluble and observed as plaques in AD.<sup>8</sup> Both amyloids A $\beta$ 40 and A $\beta$ 42 have a total charge of  $-3$  due to six acidic residues, one arginine and two lysines. They contain a hydrophobic motif that can bind to proteins and membranes and facilitate oligomerization, while the hydrophilic part is in solution.<sup>80</sup> The ratio between A $\beta$ 40 and A $\beta$ 42 is important in the formation of the soluble oligomers,<sup>100</sup> which are considered the toxic forms,<sup>101–103</sup> whereas the extracellular aggregates are not directly toxic.<sup>78</sup> A dimeric species (weight of approximately 8 kDa) has been identified as particularly toxic.<sup>104</sup> *In vivo*, there may be dozens of A $\beta$  species, and consideration of possible molecular weights of these has recently been given.<sup>69</sup> A $\beta$ 42 is most likely more toxic than A $\beta$ 40,<sup>105</sup> possibly because of the two additional hydrophobic amino acids.<sup>106,107</sup> Recently, a number of crystal structures of interactions between amyloids and various binders have been discussed.<sup>108</sup>

Several pathogenic mechanisms of soluble A $\beta$  oligomers have been given:<sup>78,109–111</sup> (i) they may damage neurons directly causing neuron death,<sup>112,113</sup> possibly upon phagocytosis<sup>114</sup> and possibly after direct A $\beta$ -generated oxidative insults;<sup>115</sup> (ii) they may destroy electrochemical signaling,<sup>116</sup> for example, by forming small membrane-soluble channels that impair ion gradients, notably Ca<sup>2+</sup>,<sup>117,118</sup> or by disrupting copper-mediated prion-protein interaction with the NMDA receptors,<sup>119</sup> impairing neuronal signaling and causing neuronal death;<sup>120,121</sup> (iii) A $\beta$  may accumulate in mitochondria<sup>122,123</sup> and disturb the respiratory chain, which then indirectly causes

oxidative stress (possibly via superoxide from inefficient respiration<sup>124</sup>), and neuronal death.<sup>125</sup>

New drugs are currently being developed that attempt to prevent the formation of toxic amyloid oligomers,<sup>126</sup> for example, inhibitors of  $\gamma$ -secretase<sup>127–129</sup> or  $\beta$ -secretase,<sup>130,131</sup> control of osmolytes that have been shown to affect amyloid formation,<sup>132–134</sup> or other types of drugs that function as  $\alpha$ -secretase enhancers either by inhibiting the proteases that degrade  $\alpha$ -secretases or by otherwise enhancing their activity or lifetime.<sup>135</sup> Readers are referred to the above references for details on the pharmaceutical targeting of the A $\beta$  production–clearance imbalance.

## 4. METAL ION HYPOTHESIS

### 4.1. The Justification of the Metal Ion Hypothesis

The lack of clinical success of anti-amyloid drugs has led some researchers to call for expansion or modification of the amyloid cascade hypothesis.<sup>65,136,137</sup> Various anomalies, such as the observations that neuron loss in AD is not correlated to amyloid load,<sup>138</sup> that 20–40% of cognitively normal people have enough amyloid plaques to cause AD diagnosis,<sup>9</sup> that clinical diagnosis is necessary because A $\beta$  biomarkers are insufficient for diagnosis,<sup>9</sup> and that AD begins and A $\beta$  accumulates in the hippocampus and cerebral cortex, although A $\beta$  itself is generated throughout the brain,<sup>73</sup> indicate that the pathogenesis of the amyloid cascade is poorly understood.<sup>73</sup> Furthermore, a toxic mode of A $\beta$ , while several are known and suggested, has not been found to be causative of AD. Even if apo-A $\beta$  oligomers in principle remain plausible key toxic substances in AD pathogenesis, the underlying causes for impaired A $\beta$  balance, which is now known to be substantially controlled by metal ions both at the APP and A $\beta$  processing levels,<sup>17,139</sup> must clearly be addressed.<sup>10,25,73</sup>

The metal ion hypothesis<sup>11,73</sup> was inspired by early suggestions<sup>140</sup> and later observations<sup>10,95,141–143</sup> that AD correlated with dyshomeostasis of metal ions, notably first Fe, and later Zn and Cu.<sup>10,144,145</sup> Iron levels have been reported to be higher in AD neuropils vs healthy neuropils<sup>146–148</sup> (the region between neurons where synaptic connections form), and iron is abnormally concentrated (millimolar concentrations) in amyloid plaques.<sup>148</sup> Magnetic resonance imaging can be used<sup>149,150</sup> to show that upon cerebral amyloid angiopathy, a lesion commonly associated with AD, the nonheme iron pool (i.e., free Fe<sup>3+/2+</sup>) is increased. Furthermore, Cu levels are generally reported to be depressed in AD brain tissue.<sup>69,151–153</sup> Currently, several genetic risk factors have also been connected to metal ion homeostasis, notably presenilin linked to calcium homeostasis and recently also to copper and zinc transport,<sup>154</sup> and mutants of the recently identified Picalm gene,<sup>49–52</sup> coding for phosphatidylinositol-binding clathrin assembly protein, are known to cause iron homeostatic deficiencies in mice.<sup>155</sup> Also, it is now clear that the central protein in the amyloid cascade, APP, is in fact regulated by and reacting with metal ions that affect amyloid balance, as will be discussed in detail below.

While discussing the metal ions, a distinction between two pools of metal ions will be made, namely “free” (i.e., loosely bound), often solvent-exposed, and mainly chelatable M<sup>2+</sup>/M<sup>3+</sup> vs strongly bound M(II)/M(III) in proteins. As will be clear later, this distinction is suggested to be crucial for understanding the pathogenesis of AD. To render the distinction semiquantitative, an approximate threshold for the dissociation constants,  $K_d \approx 10^{-7}$  M, will be used. The bound pool of M(II)

thus refers to metal ions bound to peptides and proteins with a  $K_d < 10^{-7}$  M, common for buried, specific metal-binding sites in peptides and proteins, whereas the free pool implies  $K_d > 10^{-7}$  M, which is common for metal ions binding solvent-exposed amino acid residues on surfaces of proteins.<sup>136</sup>

While much focus has been on the homeostasis of Cu<sup>17,157,158</sup> and Fe<sup>159–161</sup> in AD,<sup>162,163</sup> recent attention has been directed toward Zn in AD,<sup>10,25,164</sup> which is the center of focus of this review. Zinc content has been reported to be abnormally high in blood<sup>165</sup> and hippocampus<sup>166</sup> of AD patients. However, in the cerebrospinal fluid, zinc levels seem to be lowered in AD patients,<sup>167</sup> and globally in the brain, zinc levels have been reported to be unchanged<sup>152</sup> or reduced<sup>168,169</sup> in AD. Thus, zinc levels in AD are debatable,<sup>170</sup> and there is substantial heterogeneity in the reported zinc levels,<sup>152</sup> possibly due to sample heterogeneity, variable attention to free, chelatable Zn<sup>2+</sup> vs protein-bound Zn(II) pools, and redistributions within the brain as a function of disease progression and age. Thus, in the neocortex, the outer layer of the cerebral cortex sheet that covers the brain and is involved in learning and memory, there is disagreement between reports, some concluding that all metal ion levels including zinc are elevated<sup>171</sup> and some reporting no significant changes in overall zinc levels.<sup>152</sup> However, there is consensus that zinc is abnormally distributed in AD patients, with more zinc retained inside tissue and neurons, in particular in the synaptic vesicles,<sup>10,170</sup> and more zinc retained in amyloid plaques, consistent with elevated expression of neuronal Zn transporters (ZnT) ZnT4 and ZnT6 in early AD.<sup>10,172–174</sup> In a recent meta analysis, both iron and zinc were found to be more concentrated in certain parts of the brain such as the putamen,<sup>152</sup> which is strongly reduced in size in AD.<sup>175</sup>

A critical breakthrough for the metal ion hypothesis came from multiple independent observations that Zn(II) and Cu(II) are essential for formation and structural integrity of amyloid aggregates, oligomers, and fibrils,<sup>176–184</sup> as reviewed recently,<sup>185</sup> while normal physiological metal ion concentrations are not high enough to induce aggregation.<sup>186</sup> Furthermore, morphological evidence shows that ZnTs are necessary for plaque formation.<sup>187–189</sup> The Zn(II)-amyloids themselves are usually thought to be nontoxic, whereas Cu(II)-amyloids are neurotoxic,<sup>190</sup> although it has been reported that addition of free (i.e., in salt form) Zn<sup>2+</sup> to A $\beta$  stabilizes intermediates that lead to toxic oligomers on millisecond time scales, that is, Zn<sup>2+</sup> may take part in the formation of the toxic oligomers.<sup>191</sup> Structural interactions of both copper and zinc with amyloids have been described in great detail.<sup>176,178–183,192–194</sup> It is now accepted that amyloidosis is not spontaneous but requires metal ions for initiation.<sup>181,195</sup> These findings have been accompanied by similar discoveries of the roles of metal ions in protein misfolding, for example, relating to Parkinson's disease.<sup>196–198</sup>

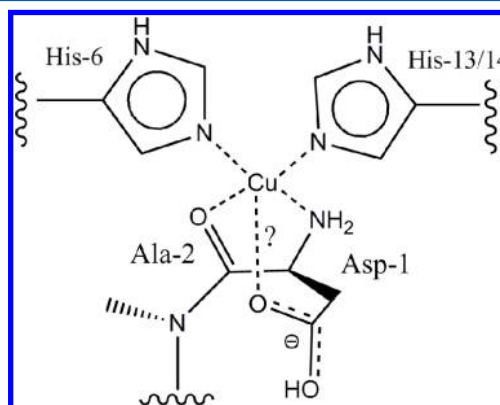
#### 4.2. Coordination Structures of A $\beta$ -Metal Complexes

The structures of Zn(II)-A $\beta$ <sup>192,199,200</sup> and Cu(II)-A $\beta$ <sup>201</sup> have been investigated in great detail by NMR,<sup>200,202,203</sup> X-ray absorption spectroscopy,<sup>204,205</sup> and Fourier transform infrared spectroscopy.<sup>192,206</sup> Both Cu(II) and Zn(II) are borderline hard-soft Lewis acids, with affinity toward N, S, and O ligands. In the free Cu<sup>2+</sup> and Zn<sup>2+</sup> forms, both ions will have a typical coordination number of 6, either fully hydrated as hexaqua ions or loosely bound on the surface of proteins, several with typical  $K_d > 10^{-6}$  M. In the bound Cu(II) and Zn(II) forms on protein active sites or specific regulatory sites,  $K_d$ 's can be much smaller

(*vide infra*) and coordination numbers may often be smaller than 6, due to the strain imposed by the peptide backbone, the entropy release due to the chelate effect on binding a peptide chain, and the basicity of involved amino acid ligands.

Most significantly, electron paramagnetic resonance (EPR) studies have been useful in elucidating the structures of paramagnetic d<sup>9</sup> Cu(II)-A $\beta$ ,<sup>185,207–209</sup> in particular when using site-specific isotopic labeling to deduce coordination modes as previously done with prion protein.<sup>210</sup> Given the dynamic structural interconversions of the Jahn-Teller distorted d<sup>9</sup> metal ion Cu(II) in water,<sup>211</sup> dynamic coordination geometries in Cu(II)-amyloids causing several types of reported coordination modes<sup>185,212</sup> are understandable, and Cu(II) geometries will thus be tetragonally distorted octahedral (coordination number 6) or trigonal bipyramidal (coordination number 5).

Cu(II) normally binds A $\beta$  in a 1:1 stoichiometry,<sup>213</sup> possibly with the existence of a second, low-affinity binding site,<sup>214</sup> and the second site may be destroyed by steric crowding in A $\beta$ 42 but be intact in shorter peptides.<sup>215</sup> The dominating binding site, located at residues 1–16,<sup>216</sup> changes with pH. At physiological pH, ~6–7, component I dominates,<sup>183,217</sup> whereas at higher pH, component II dominates.<sup>185,218</sup> Furthermore, the physiologically important component I of Cu(II)-A $\beta$  consists of at least two species in equilibrium, components Ia and Ib,<sup>208</sup> and possibly a minor third component.<sup>219</sup> The current view from labeled EPR studies is that these two components consist of four- or possibly five-coordinated Cu(II) with two imidazole nitrogens (His-6 and His-13/14 in component Ia/Ib),<sup>207,208,220,221</sup> one N-terminal amine nitrogen from Asp-1,<sup>218,222</sup> and the carbonyl oxygen of Ala-2,<sup>185</sup> possibly with a fifth, weakly bound apical/axial carboxylate from the side chain of Asp-1,<sup>192,209</sup> in contrast to previously assigned full coordination of carboxylate oxygen.<sup>201,209</sup> The consensus structure is shown in Figure 4.

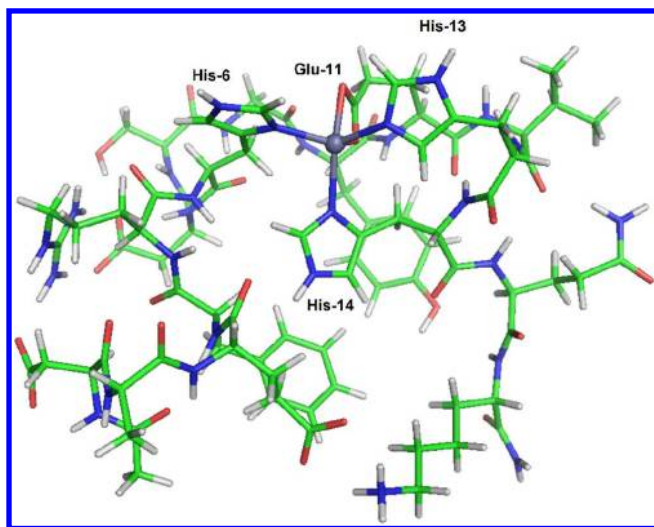


**Figure 4.** The currently most plausible first-coordination sphere structure of Cu(II)-A $\beta$  at physiological pH. Component Ia implies coordination of His-13, and component Ib implies coordination of His-14. See text for details.

Also, Arg-5 may be involved in some coordination modes,<sup>185</sup> and solvent-exposed coordination modes may occur at higher Cu(II) concentrations.<sup>223</sup> For the apparently less physiologically relevant component II, several structures have been proposed, and consensus has not yet been reached.<sup>185,207,222</sup>

In contrast, the symmetric, closed-shell d<sup>10</sup> metal ion Zn(II) binds with less structural variation for histidines and still to the same hydrophilic metal-binding 1–16 fragment of A $\beta$ , with 1:1 stoichiometry.<sup>182</sup> For solvent-exposed Zn(II), a coordination

number of six is expected, whereas in peptides and proteins, coordination numbers may be smaller as solvent exposure is reduced, that is, four or five. Whereas transition metal ions sacrifice ligand field stabilization energy upon lowering the coordination number in peptides, Zn(II) does not suffer this penalty because it is  $d^{10}$ . In  $A\beta$ , Zn(II) is found to bind to nitrogens of His-6, His-13, and His-14 without variation.<sup>199,200,224–227</sup> The remaining first coordination sphere depends on conditions. A tetrahedral geometry is possible with one additional monodentate ligand; a trigonal bipyramidal geometry is obtained when a bidentate carboxylate, such as Glu-11 (see Figure 5),<sup>200</sup> or two more monodentate ligands



**Figure 5.** The Zn(II)-binding motif of  $A\beta$ , 1ZE9.pdb. Published in ref 199. Picture produced using Pymol.

bind; and an octahedral geometry occurs if three additional ligands including solvent water bind.<sup>228</sup> Several  $A\beta$  ligands have been implied in binding Zn(II) in addition to the three histidine residues, the Asp-1 N-terminal amine,<sup>200,224,225</sup> the Glu-11 carboxylate side chain,<sup>199,200,228</sup> and the deprotonated amide of the Arg-5 backbone. Also, Tyr-10 could possibly be involved in some Zn(II) binding modes.<sup>200</sup> This would be particularly interesting in Cu(I/II) mediated redox toxicity, where tyrosine could play a role as a radical as in several copper enzymes<sup>229</sup> but is generally absent in the monomer Cu(II) structures under studied conditions, as explained above, and probably also in Zn(II)- $A\beta$  under normal conditions.<sup>230</sup>

#### 4.3. Coordination Structures of $A\beta$ Sequence Variations

Of substantial interest are structure–function correlations obtained from sequence modifications of  $A\beta$ . A main difference between rat models of AD and human AD is the lack of His-13 and Arg-5 in the rat  $A\beta$  metal-binding sequence. His-13 binds to both Cu(II) in component Ia and to Zn(II) in Zn(II)- $A\beta$ . The absence of this residue in rat amyloids reduces the metal ion affinity and leads to absence of amyloid deposits.<sup>68</sup> His-13 is, together with His-14, a target of reactive oxygen species (ROS) production in Cu(II)-amyloids,<sup>231</sup> and is essential for Zn<sup>2+</sup>-induced amyloid aggregation.<sup>232</sup>

Sequence modifications of human  $A\beta$  are also important for elucidating the binding modes of the metal- $A\beta$  complexes. While the majority of AD cases are sporadic and imply a systemic, multifactor etiology, some cases are familial.<sup>233</sup> Among these are mutations in APP and presenilin that can

affect the amyloid balance by changing the ratio between  $\beta$ - and  $\alpha$ -secretase turnover, for example, by reducing  $\alpha$ -secretase binding to the  $\alpha$ -cleavage site, or changing the  $A\beta_{42}/A\beta_{40}$  ratio,<sup>234</sup> but there are also mutations present in the actual  $A\beta$  sequence range that cause familial AD and affect the chemical properties of the produced amyloids, notably enhanced fibrillation from mutation at positions 22 and 23 such as the Dutch (E22Q), Italian (E22K), Arctic (E22G), and Iowa (D23N)<sup>235</sup> mutations that all tend to increase amyloid charge.

One mutation found to work to both effects is the APP A673V mutation that may cause AD when the mutation is homozygotic, that is, present in both APP alleles.<sup>236</sup> This APP mutation occurs in the amyloid region, at position 2 (A2V in  $A\beta$ ). EPR and hyperfine sublevel correlation (HYSCORE) spectroscopy can contribute to understanding the structural features of modified Cu(II)- $A\beta$  that enhance stabilization or toxicity.<sup>237</sup> While the first coordination sphere was found to be unaffected, there was a significant ( $\sim 0.5$ ) change in  $pK_a$  of  $A\beta$  due to the A2V mutation.<sup>237</sup> This is important because the metal binding changes with the pH (*vide infra*) and thus with the charge state of titratable ligands, which may provide a clue to the enhanced toxicity of the mutation.

Two other mutations of APP that occur in the  $A\beta$  region are the dominant “English” H6R and “Tottori” D7N mutations, associated with aggravated oligomer fibril formation toxicity.<sup>234</sup> These mutations, which like most others mentioned above increase amyloid charge, which could affect hydrophobicity, solubility, and metal-binding properties, display altered structure and Cu(II) binding and a disturbed ratio between components I and II, as evident from a range of spectroscopic methods.<sup>238</sup> Such bioinorganic chemical insight may help to explain the toxicity of  $A\beta$  mutants, and ultimately explain the pathogenesis of genetic risk factors.

Whereas apo-amyloids are negatively charged at physiological pH,<sup>239</sup> metal- $A\beta$  complexes will change the charge distribution, depending on the coordination mode and exact pH, plausibly increasing their hydrophobicity, permeability, and aggregation properties as seen for Zn(II),<sup>240</sup> which are critical to  $A\beta$ -membrane interactions.<sup>239</sup> It is in this respect notable that all the charge-increasing mutations are dominant, whereas the A2V mutant is recessive and displays different morphological and structural plaques with distinctly different distributions in the brain.<sup>241</sup> The neutralizing (increased hydrophobicity) tendency of many pathogenic mutations in the  $A\beta$  sequence of APP could be a key to understanding the pathogenesis of the produced amyloids in AD, if secretase modulation at the actual APP is not the main cause (this could be due to charge neutralization reducing, for example,  $\alpha$ -secretase  $k_{cat}/K_M$ ). Notably, a recent mutation (D7H) causing early onset AD also shares the neutralizing criterium and additionally displays enhanced metal binding properties.<sup>242</sup>

Together with the altered coordination modes of familial AD-causing  $A\beta$ -mutations, the different coordination modes of Zn(II) and Cu(II) in human  $A\beta$  could explain the different rates of their amyloid formation,<sup>243</sup> as could the different kinetics observed for Cu(II)-induced  $A\beta$  formation in rats and humans.<sup>244</sup> Also, K-edge X-ray absorption spectroscopy suggests that the coordination mode of Cu(II) differs in  $A\beta$  monomers and the assumed toxic oligomers,<sup>245</sup> an important focus to completely understand the structure–function correlations leading to metal-induced oligomerization and possibly toxicity. Furthermore, recent spectroscopic studies<sup>246</sup> on mixed Zn(II)/Cu(II)- $A\beta$  complexes conclude that Zn(II)



can disturb the Cu(II) coordination mode away from histidine-binding and thus possibly protect against a toxic Cu(II)-binding mode. Given the stronger Cu(II) binding, such a displacement is surprising, although not impossible when only some ligands are involved in the substitution. In fact, this mechanism may resemble the proposed beneficial substitution of Cu(II) for Zn(II) in Cu(II)-A $\beta$  by Zn<sub>7</sub>-metallothionein,<sup>247</sup> to be discussed later. More molecular insight into the interplay between Zn(II) and Cu(II) in amyloid binding and oligomerization is clearly warranted.

#### 4.4. The Affinities of Metal Ions for A $\beta$

A central focus of bioinorganic AD research is to systematically understand the affinities of metal ions for various targets associated with the disease, most often described by the metal dissociation constant,  $K_d$ .  $K_d$ 's are essential for the concept of free and bound metal ion pools, which may be critical to AD pathogenesis (*vide supra*), and to define potent chelation and metalloprotein inhibition therapies. The determination of accurate  $K_d$ 's by competitive ligation is quite challenging and results differ greatly depending on pH and ionic strength, unintended formation of ternary or buffer complexes, or inefficient competition.<sup>156</sup>

Thus, the reported stabilities of 1:1 metal-A $\beta$  complexes vary substantially,<sup>212,248,249</sup> with reported conditional  $K_d$ 's in the range from  $10^{-11}$  to  $10^{-7}$  M for Cu(II)-A $\beta$  (most likely consensus  $10^{-10}$  to  $10^{-9}$  M<sup>156,249</sup>) and  $10^{-9}$  to  $10^{-6}$  M for Zn(II)-A $\beta$ , with  $K_d$  values of  $\sim 10^{-7}$  M being seen in most studies,<sup>192,212</sup> although very weak  $K_d$ 's  $\approx 10^{-6}$  to  $10^{-5}$  M have also been inferred.<sup>228</sup> Cu(II) typically binds 2 orders of magnitude better than Zn(II) in these histidine-binding systems. The Irving-Williams series of increasing stability constants puts Cu(II) before Zn(II) due to its strong Jahn-Teller effect,<sup>250</sup> as also seen for oxygen ligands such as EDTA with typical  $K_d$ 's of  $10^{-19}$  and  $10^{-17}$  M for Cu(II) and Zn(II), respectively.<sup>251</sup> For many nitrogen chelators,  $10^5$ - $10^6$  orders of magnitude difference between Cu(II) and Zn(II) is common for overall association constants, whereas for peptides and proteins discussed in this review, 2 orders of magnitude difference between Cu(II) and Zn(II) is more common.

For comparison, typical concentrations of copper and zinc in AD senile plaques are  $\sim 0.4$  and  $\sim 1$  mM, respectively,<sup>148</sup> similar in fibrils and soluble oligomers.<sup>192</sup> Recently, a kinetic three-step mechanism of Cu(II)-induced oligomerization was suggested based on fluorescence and NMR spectroscopy, identifying a nonoligomeric, that is, potentially innocent, monocopper-diamyloid complex that may be targeted for preferential stabilization as a treatment strategy.<sup>195</sup>

In addition to copper and zinc, also other free metal ions such as iron or aluminum<sup>252</sup> may interact with, stabilize, or induce aggregation or oligomer formation of A $\beta$ .<sup>253</sup> On a parallel note, heme-iron homeostasis has been found to be impaired in AD,<sup>254</sup> and heme binds to A $\beta$  and inhibits both aggregation and oxidative toxicity of A $\beta$  *in vivo*.<sup>255,256</sup> Given the importance of heme homeostasis for the mitochondrial neuronal energy production and antioxidant activity, this is a significant observation that may further link metabolic deficiencies in AD to the metal ion hypothesis.<sup>255,257</sup> The same metal binding region 1-16 of A $\beta$  as involved in Cu(II) and Zn(II) binding also binds to heme. Most likely, heme binds mainly to His-13 or possibly His-14 via axial coordination to heme.<sup>258</sup> In principle, up to two histidines can coordinate at a time to generate a coordination number of 6 as in octahedral

coordination geometries of cytochromes, for example, but EPR data indicate a  $g$  value of  $\sim 6$  resembling high-spin as in pentacoordinate deoxyheme.<sup>258</sup> Interestingly, heme may out-compete Zn(II) or Cu(II) in amyloids, thus preventing oligomer formation.<sup>254</sup> As implied by other differences between rat and human A $\beta$  structures differing in metal-mediated aggregation and toxicity, heme also binds differently to human and rodent amyloids and could indicate a heme-A $\beta$ -mediated mode of oxidative toxicity in AD.<sup>259</sup>

#### 4.5. The Role of Zinc in AD

Zinc plays a central role in the central nervous system (CNS) in processes such as apoptosis, oxidative stress, and immune defense,<sup>260</sup> neurogenesis, motor coordination, memory, and synaptic plasticity.<sup>261-263</sup> Zinc dyshomeostasis is a pathological feature of AD,<sup>264-267</sup> depression,<sup>264,268,269</sup> Parkinson's disease,<sup>267,270</sup> autism spectrum disorders (ASD),<sup>271,272</sup> amyotrophic lateral sclerosis (ALS),<sup>267,273,274</sup> epilepsy,<sup>275,276</sup> and schizophrenia.<sup>264</sup>

Zn(II) is bound in more than 300 proteins,<sup>277</sup> in transcription zinc-fingers (typical  $K_d$ 's  $\approx 10^{-12}$  M<sup>278</sup> although down to  $10^{-15}$  M has been observed<sup>279</sup>), stored in metallothioneins (MT), and present as a free, chelatable Zn<sup>2+</sup> pool in the vesicles of terminals of zinc-enriched neurons (ZEN), for example, zinc- and glutamate-releasing (*gluzineric*) neurons.<sup>280</sup> The gradient of the free Zn<sup>2+</sup> pool is tightly controlled with only  $10^{-12}$  M free intracellular Zn<sup>2+</sup> and up to millimolar vesicular Zn<sup>2+</sup><sup>144</sup> by MTs for storage and buffering and by transportation across membranes via zinc transporter proteins of two families, ZnT and ZIP.<sup>281</sup> Once in the vesicles of neurons, the vesicular Zn<sup>2+</sup> is released during neurotransmission together with glutamate and modulates glutamate-activated neurotransmission via inhibition of  $\gamma$ -aminobutyric acid (GABA) and NMDA receptors<sup>10,262,264,282,283</sup> by binding specific Zn<sup>2+</sup>-binding sites in the receptors.<sup>284</sup> The *gluzineric* neurons are mainly located in the cerebral cortex, the central stage of AD, and in particular in the limbic system.<sup>264,285</sup>

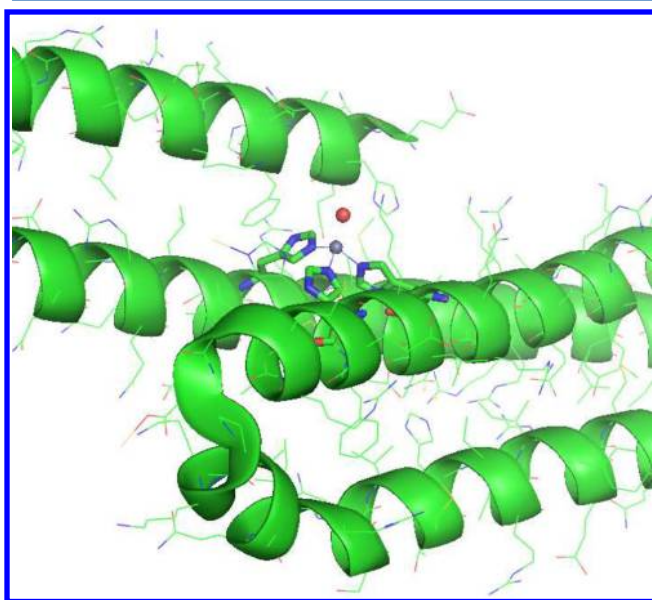
The role of zinc in AD pathogenesis, first suggested by Burnet,<sup>264</sup> is substantiated by observations that the genetic risk factor in familial AD, the  $\epsilon 4$  apolipoprotein E gene, is correlated with higher serum levels of Zn, Cu, and insulin, and that only zinc is an independent risk factor, not  $\epsilon 4$  apolipoprotein E itself.<sup>286</sup> Zinc-dependent amyloidosis can also explain some gender differences in plaque formation in APP transgenic mice,<sup>287</sup> although many other factors affect gender-specific risk factors of AD, for example, life style, genetic, and cholesterol correlations. However, evidence of zinc dyshomeostasis in AD comes from the vast number of reports describing changes in ZnT levels, zinc redistribution, and direct zinc-amyloid interactions, as described in sections 4.1 and 4.2.

Zinc affects A $\beta$  balance in several ways, both via transcription factor zinc-fingers, in regulatory Zn<sup>2+</sup> sites notably in APP, and as bound Zn(II) in active sites of zinc proteases.<sup>10</sup> First, regulatory Zn<sup>2+</sup> can directly bind and inhibit APP at the  $\alpha$ -secretase site,<sup>288</sup> leaving APP to cleavage by the other two secretases to enhance production of A $\beta$ .<sup>67</sup> Other researchers have observed reduced total A $\beta$  production but enhanced intracellular A $\beta$  production upon zinc binding to APP.<sup>289</sup> The zinc-binding site in APP has a conditional  $K_d$  of  $\sim 10^{-6}$  M,<sup>288</sup> similar to or slightly weaker than that of Zn(II)-A $\beta$ . The central AD protein APP, long obscure in its biological role, has recently been found to possess ferroxidase activity, oxidizing Fe<sup>2+</sup> to Fe<sup>3+</sup> before loading Fe<sup>3+</sup> into transferrin, and this



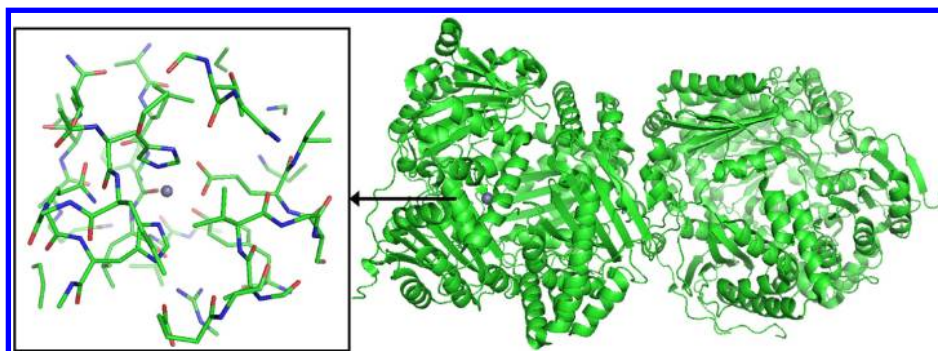
function is inhibited by  $Zn^{2+}$ .<sup>290</sup> This mechanism could provide a coupling between iron dyshomeostasis and  $A\beta$  imbalance and would make zinc dyshomeostasis a plausible cause of both.

In addition to the copper binding domain in the E1 extracellular domain of APP, to be discussed below, several metal sites in the extracellular E2 domain of APP have recently been structurally characterized by X-ray diffraction, providing evidence for Cu(II) and Zn(II) as controlling regulatory metal ions in the conformation and possibly function of this domain.<sup>139</sup> Two intramolecular metal binding sites and several solvent-exposed sites were observed, with the high-affinity M1 site consisting of four histidines (APP sequence numbers His-313, His-382, His-432, and His-436) binding to Cu(II) in a Jahn–Teller distorted square planar geometry. In case of Zn(II), His-313 is substituted for a water ligand (see Figure 6).



**Figure 6.** M1 binding site in the amyloid precursor protein (APP) extracellular E2 domain, occupied by Zn(II). PDB code 3UMI. Picture produced with Pymol.

In the Cu(II) form, two  $\alpha$  helices are bridged by the metal ion, whereas in the Zn(II) form, since His-313 is located on a separate  $\alpha$  helix, there is no direct bridge. The  $K_d$ 's for the site were found to be  $\sim 10^{-8}$  and  $\sim 4 \times 10^{-6}$  M for Cu(II) and Zn(II), respectively, in good agreement with previously determined values.<sup>287</sup>



**Figure 7.**  $Zn^{2+}$ -binding site in the human amyloid-degrading protease, insulin-degrading enzyme. PDB-code 2G54.pdb. From ref 292. Picture made with Pymol.

Zn(II) is also required in the active site of the ADAM family of  $\alpha$ -secretases to catalyze the nonamyloidogenic cleavage, as shown in Figure 3.<sup>8,291</sup> Furthermore, free  $Zn^{2+}$  also inhibits matrix metalloprotease-2, neprilysin, and insulin-degrading enzyme (see Figure 7),<sup>292</sup> which degrade  $A\beta$ .<sup>94</sup> The extracellular metalloproteinases are expressed in the astrocytes, to be discussed further below.<sup>94</sup>

Regarding neurotransmission and cognition, free  $Zn^{2+}$  is known to inhibit GABA receptors,<sup>10,293</sup> thus modulating their  $Cl^-$ -mediated hyperpolarization, facilitating controlled neurotransmission and preventing excitotoxicity.<sup>10</sup> It is the Zn transporter ZnT3 that loads  $Zn^{2+}$  into the synaptic vesicles where it is again released during neuromodulation.<sup>68</sup> This is the likely reason excessive free  $Zn^{2+}$  causes seizures and may be a trigger of epilepsy.<sup>294–296</sup> Mice without ZnT3 still accumulate free neurotoxic  $Zn^{2+}$  from intracellular stores not associated with the synaptic vesicles, where the ZnT3 is located, indicating a key role of intracellular zinc buffering proteins, that is, metallothioneins, in neurodegenerative zinc dyshomeostasis.<sup>297</sup> Excess free  $Zn^{2+}$  will ultimately lead to neuronal necrosis or apoptosis.<sup>10,264,298–300</sup> Excess free  $Zn^{2+}$  also induces phosphorylation of tau-protein<sup>301</sup> and remains located in neurites with neurofibrillar tangles.<sup>302</sup>

#### 4.6. The Role of Copper in AD

Copper homeostasis is of vital significance to the brain<sup>303,304</sup> and is also impaired in AD as in other neurological disorders.<sup>305–308</sup> Copper homeostasis is extremely critical, because concentrations of free  $Cu^{2+}$  beyond  $10^{-18}$  M may cause oxidative damage.<sup>309</sup> The most common transported form of copper is instead Cu(I).<sup>310</sup> Copper in both oxidation states is located in active sites of a number of vital redox-active proteins such as ceruloplasmin, cytochrome *c* oxidase, prion protein, tyrosinase, and Cu,Zn-superoxide dismutase (cytoplasmic SOD-1 and extracellular SOD-3), and its absence from these proteins is dramatic, as seen from neuronal degeneration in Menkes disease caused by lack of copper transport across the blood–brain barrier (BBB).<sup>304</sup>

While total Cu levels are mostly observed to be depressed in AD,<sup>152,153</sup> some reports show increased levels<sup>148,253</sup> and some reduced levels in AD brains (hippocampus/amygdala),<sup>311</sup> probably reflecting the loss of bound pools, subsequent redistribution to extracellular space,<sup>69,312</sup> specifically to  $A\beta$  in the cerebrospinal fluid<sup>313</sup> and eventually to the blood serum.<sup>314</sup> Thus, the emerging consensus of copper seems consistent with that of zinc, namely, relocation from intracellular to extracellular stores and from bound to free pools present in

serum or in A $\beta$  deposits, as seen in mouse models of AD,<sup>315</sup> although total Cu levels are also generally depressed.<sup>152,153</sup> This picture of temporal and spatial changes in the two pools is important for the design of proper biomarkers and for discussing metal ion levels in neurological disease, including AD.

The uptake, storage, transport, and transfer of Cu(I/II) to proteins depend on a range of copper transporters and chaperones.<sup>316</sup> After being mostly absorbed in the liver, it is incorporated into copper proteins (notably ceruloplasmin discussed in section 4.8) by chaperones. To reach the brain, copper is transported first across the BBB via copper pumps (copper ATPases ATP7A and ATP7B)<sup>317,318</sup> with the possible help of Atox1, which may work as a Cu(I) chaperone for ATP7A<sup>319</sup> and has been reported to facilitate antioxidant function in neurons<sup>320</sup> and cell growth.<sup>321</sup> Subsequently, copper is taken up by the copper transport protein Ctr1<sup>322–324</sup> and distributed to its various destinations in the neurons, notably in the plasma membrane and in vesicles.<sup>310</sup> Ctr1 and the copper ATPases are regulated by the copper concentration, and copper binding in regulatory sites promotes endocytosis and degradation of Ctr1 to prevent further copper uptake and intraneuronal copper accumulation.<sup>310,325</sup>

Copper binds to prion protein and ceruloplasmin (90% of all blood Cu<sup>144</sup>), and a significant part is kept in the brain in MTs,<sup>305,326,327</sup> particularly inside the astrocytes, mainly in the Cu(I) form.<sup>316</sup> Some of this Cu(I) from MT has been shown to be transferred to SOD *in vitro*, although not *in vivo*,<sup>327</sup> and Cu-containing MT-3 is likely to act as a Cu-transferring chaperone for several Cu proteins.<sup>328,329</sup> Various other chaperones transfer copper to critical proteins such as antioxidant SOD (copper-chaperone for superoxide dismutase, CCS)<sup>309,330</sup> and respiratory cytochrome *c* oxidase (Cox17).<sup>331,332</sup> Mutations or post-translational modifications in these proteins could in principle impair copper homeostasis in the brain causing sporadic risk of neurological disease, CCS for ALS and Cox17 for mitochondrial impairment seen in most neurological disorders.

The loss of protein-bound Cu(II)/Cu(I) probably reflects reduced function of copper proteins, but whether this loss is a cause or effect of neuron degeneration (e.g., following apoptotic events) must be investigated. For example, recent work suggests a new toxic mode of A $\beta$  via interference with copper/prion-protein modulation of NMDA receptors that would interrupt Ca<sup>2+</sup>-dependent neurotransmission.<sup>119</sup> Disrupted copper active sites are a smoking gun in neurodegeneration, because they lead to neurological disorders such as ALS (Cu,Zn-SOD-1 by gain of toxic function<sup>333</sup>), Parkinson's disease ( $\alpha$ -synuclein),<sup>90,91</sup> or Menkes and Wilson disease (copper ATPases ATP7A and ATP7B, respectively)<sup>305,306</sup> and may further explain impaired mitochondrial energy production (i.e., low glucose uptake in brain disorders), for example, via Cu- and Fe-containing cytochrome *c* oxidase. Cytochrome *c* oxidase is also known to be inhibited by free Zn<sup>2+</sup>,<sup>334,335</sup> providing an example where metal dyshomeostasis may converge to impair mitochondrial energy production.

In addition to loss of bound Cu(I)/Cu(II), several pathogenic mechanisms of free Cu<sup>+</sup>/Cu<sup>2+</sup> are known:<sup>336</sup> Cu<sup>2+</sup> may bind to APP, possibly at a site involving His-147, His-151, and Tyr168,<sup>337</sup> and initiate oxidative stress via reduction to Cu<sup>+</sup> and subsequent formation of hydroxyl radicals.<sup>338</sup> The Cu(II) binding site in APP has a  $K_d$  of  $\sim 10^{-8}$  M,<sup>339</sup> which is reasonable given that the Zn(II) binding site has an estimated  $K_d \approx 10^{-7}$  M.<sup>288</sup> Intracellular reductions in Cu availability, as observed in

AD,<sup>69,151,153</sup> lead to enhanced production of A $\beta$ , providing one more cause of amyloid imbalance,<sup>340</sup> and regulation of APP via this site is a possible mode for this.

In addition to post-translational modification of APP, Cu(II)-A $\beta$  also produces ROS by itself, for example, peroxide formation<sup>341</sup> and lipid peroxidation initiated by Cu(II)-induced dityrosine formation from Tyr-10.<sup>342</sup> It is important in this context that Cu-A $\beta$  is more toxic than apo-A $\beta$ ,<sup>341,343,344</sup> and Cu(II) may confer toxicity to the A $\beta$  in a concentration-dependent manner.<sup>345,346</sup>

A $\beta$  oligomers may enhance permeability of neuron and organelle membrane where APP is located and cause membrane dysfunction disrupting homeostasis.<sup>347</sup>  $\gamma$ -Secretase has been found in the mitochondrial membranes<sup>348</sup> and intracellular A $\beta$ <sup>8</sup> produced in this way could penetrate the mitochondrial barrier from the cytoplasm in synaptic terminals where APP and amyloids are enriched.<sup>347</sup> Amyloids can also accumulate inside the mitochondria as seen in APP-over-expressing mice.<sup>123,349</sup> Inside the mitochondria, amyloids disturb normal mitochondrial functions in a variety of ways leading to Ca<sup>2+</sup> dyshomeostasis and apoptosis.<sup>350</sup>

Whether the membrane toxicity of amyloid oligomers is the critical toxicity in AD and, if so, whether it involves or even requires metal-A $\beta$  complexation in the toxic form is unknown, and such information could potentially help to reconcile the amyloid-cascade and metal-ion hypotheses. However, the two hypotheses are also consistent absent a toxic metal-A $\beta$  mode given that metal ions remain necessary for induction of toxic oligomer formation and APP regulation. Still, given the role played of hydrophobicity in membrane interactions of amyloids,<sup>239</sup> a membrane-toxic mode of metal-oligomers is plausible given the likely enhancement of hydrophobicity caused by partial charge neutralization of the negatively charged A $\beta$  upon metal binding, which could render amyloid oligomers less amphiphilic membrane-binding and more membrane-penetrating. Supporting the role of hydrophobicity as a toxic property (by oligomerization propensity or membrane permeability), many familial AD mutations are charge-neutralizing as discussed in section 4.3, and the A $\beta$ 42 contains two more hydrophobic residues at the C-terminal (Figure 2). In contrast, the apo-amyloids could in fact function as antioxidants during normal metal homeostasis.<sup>80,336</sup>

#### 4.7. The Role of Calcium in AD

In this review, divalent calcium is written exclusively as Ca<sup>2+</sup> instead of Ca(II) to mark it as a mainly free, often hydrated and labile, redox-inactive metal ion with dissociation constants ranging between 10<sup>-9</sup> and 10<sup>-4</sup> M,<sup>351</sup> but typically in the micromolar range as seen for calmodulin.<sup>352,353</sup>

In the healthy cell, the cytosolic concentration of Ca<sup>2+</sup> is maintained close to 100 nM, 4 orders of magnitude lower than extracellular concentrations ( $\sim 2$  mM), due to Ca<sup>2+</sup> storage proteins such as calbindin and by active transport via Ca<sup>2+</sup>-ATPases.<sup>354,355</sup> Disturbance of this homeostasis will affect mitochondria that utilize Ca<sup>2+</sup> in energy production, specifically enzymes such as pyruvate dehydrogenase, isocitrate dehydrogenase, and ATP synthase, which are all regulated by Ca<sup>2+</sup>.<sup>355</sup> If Ca<sup>2+</sup> concentration is abnormally high inside mitochondria, it becomes a main cause of neurotoxicity.<sup>356</sup> Glutamatergic neurons with impaired mitochondria produce too little ATP and may not be able to retain the membrane potential needed to allocate Mg<sup>2+</sup> to NMDA receptors, leaving them chronically

open to  $\text{Ca}^{2+}$  and causing excitotoxicity, mitochondrial membrane permeability, and apoptosis.<sup>70,357</sup>

The involvement of calcium dyshomeostasis in AD has been known for more than 2 decades<sup>358</sup> and has been reviewed recently by several authors.<sup>359–363</sup> Age-correlated gene expression related to calcium homeostasis has been shown to be magnified in AD.<sup>364</sup> The role of calcium dyshomeostasis in AD has been emphasized via mutants in presenilin, a key risk factor in AD and part of the  $\gamma$ -secretase complex,<sup>365,366</sup> and increased intraneuronal  $[\text{Ca}^{2+}]$  is correlated with APP mutations, ApoE4 expression, tau hyperphosphorylation, and  $\text{A}\beta$ -plaque formation.<sup>117,367</sup> Recent genetic risk of AD was further associated with the calcium homeostasis modulator 1, confirming this relationship.<sup>368,369</sup> The possible roles of presenilin in calcium transport and homeostasis have recently been reviewed.<sup>367,370</sup> Notably, presenilin appears to function as a calcium leak channel independently of the activity of the enzyme complex  $\gamma$ -secretase of which presenilin is also a part.<sup>370</sup>

Impaired calcium homeostasis provides the simplest possible explanation (in the Occam's Razor sense) of reduced synaptic plasticity and memory deficits in AD,<sup>371</sup> where other hypotheses must identify new modes of impaired memory formation and maintenance. Furthermore, apoptosis, one of the programmed cell death processes that terminate many neurological diseases including AD, depends directly on calcium homeostasis. Caspases that mediate apoptosis are  $\text{Ca}^{2+}$  dependent and are found to be activated in aging people and more so in AD.<sup>76,372</sup> Calcium dyshomeostasis is further aggravated by  $\text{A}\beta$ , which may increase  $\text{Ca}^{2+}$  permeability.<sup>373</sup> This may be a primary toxicity of  $\text{A}\beta$  and would imply that calcium dyshomeostasis is a consequence of previous  $\text{A}\beta$  imbalances.<sup>117,120,374</sup>

Many of the pathogenic aspects of calcium correlate with and perpetuate those of zinc due to the interplay between these two metal ions in glutamate-controlled neurotransmission<sup>354</sup> and in membrane transport,<sup>375</sup> for example,  $\text{Zn}^{2+}$  transport to mitochondria mainly occurs via the  $\text{Ca}^{2+}$  uniporter.<sup>144</sup> Also, as mentioned above,<sup>119</sup>  $\text{A}\beta$  may disrupt Cu–prion-protein mediated NMDA receptors involved in  $\text{Ca}^{2+}$  signaling, linking the calcium dyshomeostasis and neurotoxicity observed in AD directly to zinc, copper, and  $\text{A}\beta$ .

#### 4.8. The Role of Iron in AD

Iron homeostasis is critical to the CNS, as evidenced from iron dyshomeostasis observed in neurodegeneration.<sup>376–383</sup> Iron is as mentioned also dysregulated in AD<sup>14,15,159,160,384</sup> with abnormally distributed iron pools in AD,<sup>385</sup> notably an increased pool of free, nonheme iron  $\text{Fe}^{3+}/\text{Fe}^{2+}$  and functional heme deficiency.<sup>151,386</sup> In fact, heme degradation has been suggested as a biomarker for early detection of AD<sup>387</sup> and dysregulated free iron pools have been suggested as a main cause of several neurological disorders.<sup>377</sup> Section 4.2 described the specific interactions between heme and amyloids that could be a consequence of this dysregulation. Also, transferrin levels have been found to be depressed in AD.<sup>388</sup>

Iron homeostasis is normally governed by a range of proteins such as ferritin and transferrin,<sup>389</sup> which store ~25% of the body's iron, mainly as ferric  $\text{Fe}^{3+}$ , and have been implicated in neurodegeneration,<sup>390</sup> and by the heme-pool, that is, mainly ferrous iron bound to heme proteins such as hemoglobin and myoglobin, which store more than half of the total iron,<sup>391</sup> the heme carrier protein 1,<sup>392</sup> and the heme-degrading enzyme

heme oxygenase (HO), which is also implicated in AD.<sup>393,394</sup> The peptide hormone hepcidin plays a key role in iron regulation<sup>159</sup> and regulates, for example, divalent metal transporter 1 (DMT-1), which plays a significant role in transporting not just iron but also copper<sup>395</sup> and has been implied in Parkinson's disease<sup>396</sup> and as a transporter of the metal ions that regulate the processing of APP and thus amyloid production.<sup>397</sup> Neurons express transferrin and DMT-1 but generally little ferritin, suggesting that the iron pool of free  $\text{Fe}^{3+}/\text{Fe}^{2+}$  is tightly controlled, and that iron is immediately recruited by neurons when required.<sup>383</sup> This recruitment requires reduction of  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  before binding to DMT-1.<sup>383</sup>

Iron is responsible for  $\text{O}_2$ -storage in hemoglobin and myoglobin and is necessary for mitochondrial  $\text{O}_2$ -dependent energy production via a large number of enzymes, for example, NADH dehydrogenase (complex 1), cytochrome *bc*<sub>1</sub> (complex 3), and cytochrome *c* oxidase (complex 4) in the mitochondrial electron transport chain. Thus, impaired Fe/heme metabolism may play a role in metabolic deficiencies observed during AD pathogenesis.<sup>259</sup> Iron is also in the active sites of several enzymes involved in synthesis of neurotransmitters, for example, tryptophan hydroxylase catalyzing the first step of serotonin and melatonin synthesis.<sup>398</sup>

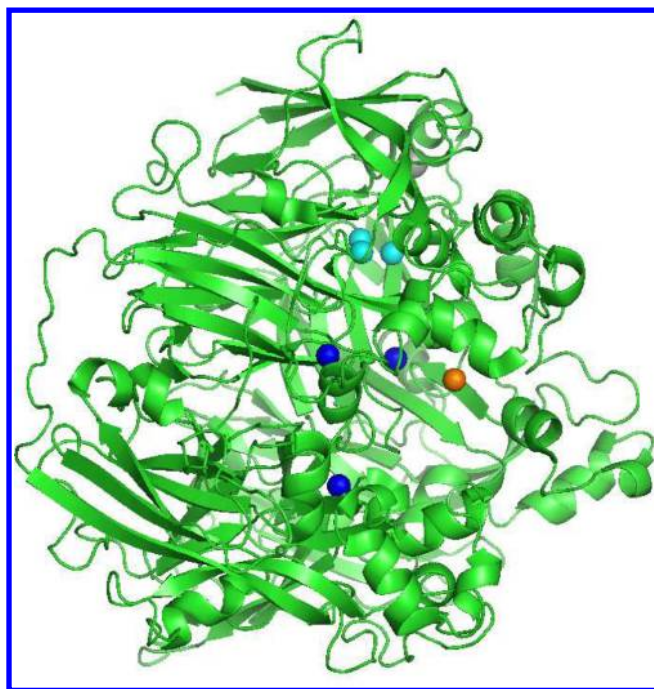
Iron accumulates in the aging brain and enhances the overall oxidative stress level.<sup>399</sup> Heme dyshomeostasis is evident in AD from the interaction of heme oxygenase with neurofibrillar tangles,<sup>400</sup> reactive astrocytes, and senile plaques in AD brain tissue<sup>401</sup> and from reduced activity of heme oxygenase in the Swedish AD-causing APP mutants related to mutant APP disrupting heme oxygenase activity.<sup>402</sup> In AD, iron-caused ROS production is evident.<sup>403,404</sup> Toxic concentrations of iron are found in extracellular  $\text{A}\beta$  of AD patients,<sup>147,381</sup> testifying to its role in plaque formation as a possible consequence of elevated free iron concentrations.<sup>178</sup> In AD hippocampus and cerebral cortex, this dysregulation is partly compensated by elevated levels of iron storage proteins.<sup>405</sup>

In terms of toxic mechanism, free  $\text{Fe}^{3+}$  and  $\text{Al}^{3+}$  have been found to induce tau protein aggregation, whereas divalent metal ions did not.<sup>406</sup> Overexpression of heme oxygenase causes tau phosphorylation and aggregation in mouse brains.<sup>407</sup> Iron/heme homeostasis is also critical to antioxidant activity via enzymes such as catalases and peroxidases, and functional heme deficiency, symptomatic by heme binding to amyloids and the up-regulation of heme synthesis in AD,<sup>386</sup> could arise from the observed heme interaction with  $\text{A}\beta$ , causing direct oxidative stress.<sup>254,257,255,259</sup>

Whether iron dyshomeostasis occurs before other dyshomeostasis as suggested by some authors<sup>386,408</sup> remains to be established, but the homeostasis of various metal ions are in fact intimately related. An important example of this is ceruloplasmin,<sup>409</sup> suggested to play an important role in AD.<sup>410,411</sup> It is a six-domain multicopper oxidase with the structure shown in Figure 8 (2JSW.pdb),<sup>412</sup> with three mononuclear T1 copper sites (dark-blue) and a trinuclear combined T2/T3 copper site (cyan). An additional, solvent-exposed transition metal site, possibly where a substrate  $\text{Fe}^{2+}$  binds, is shown in orange, and a  $\text{Ca}^{2+}$  site is shown in gray.

Ceruloplasmin carries most of the copper in the serum<sup>413</sup> and oxidizes several substrates by electron abstraction and transfer to  $\text{O}_2$  bound by copper at the T2/T3 site, which is four-electron reduced to produce water as in other multicopper oxidases.<sup>414–416</sup> Among its substrates is  $\text{Fe}^{2+}$ , that is, ceruloplasmin functions as a ferroxidase,<sup>417,418</sup> oxidizing  $\text{Fe}^{2+}$





**Figure 8.** The structure of the multicopper oxidase ceruloplasmin at 2.8 Å resolution (2JSW.pdb),<sup>412</sup> with its six active site coppers shown in dark blue (mononuclear T1 sites) and cyan (trinuclear O<sub>2</sub>-reducing T2/T3 site). The additional, solvent-exposed transition metal site is shown in orange, and the Ca<sup>2+</sup> site is shown in gray (Picture made with Pymol).

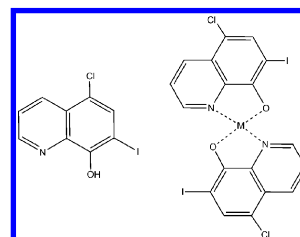
to Fe<sup>3+</sup>. Ceruloplasmin has an antioxidant function *in vivo*<sup>419–421</sup> and has been linked to neurological disease.<sup>422,423</sup> Mutations in ceruloplasmin can lead to loss of catalytic function resulting in a disease known as aceruloplasminemia, where iron accumulates in neurons, causing diabetes and neurodegeneration,<sup>424</sup> oxidative stress, motor deficits, and dementia.<sup>425,426</sup>

Ceruloplasmin is expressed in neurons<sup>423</sup> and is membrane-anchored in astrocytes<sup>427</sup> that are central to the brain's homeostasis, including metal and oxidative stress control, express the extracellular metalloproteases that degrade amyloids,<sup>94</sup> and may be dysfunctional during neuronal degeneration.<sup>428,429</sup> It is plausible that membrane ceruloplasmin is required for immediate oxidation of Fe<sup>2+</sup> to Fe<sup>3+</sup> as required by transferrin,<sup>427</sup> before Fe<sup>2+</sup> otherwise engages in toxic Fenton chemistry (*vide infra*). Thus, lack of catalytic function of ceruloplasmin, for example, due to absence of copper transfer from dysfunctional astrocyte MT-3 or modification of ceruloplasmin itself, as recently seen with carbonylation from oxidative stress correlating with Parkinson's disease,<sup>430</sup> could lead to accumulation of toxic Fe<sup>2+</sup> and associated oxidative stress and iron dyshomeostasis. To support this hypothesis, increased apo-ceruloplasmin levels and decreased ceruloplasmin activity has been observed in AD patients.<sup>431</sup> Ceruloplasmin deficiency, probably via iron deficiency in tryptophan hydroxylase, also produces serotonin deficiency.<sup>432</sup> This could also explain the decreased transferrin levels observed in AD.<sup>388</sup> If so, the ferroxidase activity of ceruloplasmin comes into close pathogenic relationship with APP, which was recently shown to regulate iron homeostasis by ferroxidase activity in a similar way, and this function was inhibited by Zn<sup>2+</sup>.<sup>290</sup> In these cases, Cu/Zn dyshomeostasis would then be a causal factor in both iron dyshomeostasis, oxidative stress, and the amyloid cascade.

#### 4.9. The Quest for Metal-Chelating AD drugs

Because of the discussed recent breakthroughs in AD research, bioinorganic chemistry is entering the AD field in full force, and metal chelators are being rapidly developed for possible treatment of AD.<sup>18,73,433–450</sup> Some of the therapeutic aspects associated with chelation therapy in AD have recently been reviewed.<sup>451,452</sup>

Particularly notable in this development was clioquinol<sup>453</sup> (iodochlorhydroxyquin; see Figure 9), an anti-infectious drug



**Figure 9.** Clioquinol, left, and an example of its coordination to metal ions, right.

that is also a Zn- and Cu-chelator<sup>77</sup> that reduces A $\beta$  load in AD patients, and its derivatives.<sup>454,455</sup> Clioquinol has been suspected of causing subacute myelo-optic neuropathy (SMON) in Japan after massive use during the 1960s, although the direct causality has been debated.<sup>456</sup> Because SMON affects the spinal cord, eyes, and peripheral nerves and leads to blindness and paralysis, it is plausible that overdoses of clioquinol, if responsible, impair metal homeostasis in the CNS, leading to the observed symptoms. Thus, the SMON cases are important reminders of the danger associated with application of chelators to rebalance metal homeostasis, because this treatment is constrained by a narrow therapeutic window determined by the  $K_d$  range that specifically binds the target without stripping metal ions from vital enzymes.

Sometimes, chelators such as clioquinol are referred to as metal–protein-attenuating compounds (MPAC) assuming that they cause metal release from peptides and resumption of A $\beta$  clearance.<sup>457</sup> If so, competitive binding requires the chelator to have higher metal ion affinity than A $\beta$ ; for Cu(II) and Zn(II),  $K_d$  is typically  $\sim 10^{-10}$ – $10^{-9}$  M and  $\sim 10^{-7}$  M, respectively, depending on concentrations and pH.<sup>192</sup> As discussed recently,<sup>192</sup> an effective MPAC should then have a  $K_d$  of perhaps  $\sim 10^{-10}$  M to release Cu(II) from A $\beta$ , or  $10^{-8}$  M to strip Zn(II), but should not strip Cu(II) or Zn(II) from systemic sites ( $< 10^{-10}$  M, e.g.,  $10^{-12}$  M for serum albumin<sup>458</sup>). The  $K_d$ 's of 1:2 metal–clioquinol complexes at neutral pH are  $\sim 10^{-10}$  M for Cu(II) and  $\sim 10^{-9}$  M for Zn(II).<sup>459</sup> The 1:1 and 1:2 complexes of Cu(II) and Zn(II) with clioquinol display distorted tetragonal and trigonal bipyramidal coordination geometries, respectively.<sup>460</sup> Although these were achieved at very high clioquinol concentrations, they suggest that clioquinol can work directly as an MPAC.

Copper-binding proteins with multiple binding sites involved in other neurological diseases display  $K_d$ 's from the  $10^{-18}$  M range ( $\gamma$ -synuclein<sup>461</sup>) to the  $10^{-10}$ – $10^{-9}$  M range (Parkinson's disease-related  $\alpha$ -synuclein<sup>462</sup>). The octarepeat copper binding site in prion protein, which involves histidine coordination, may also have a  $K_d$  of  $\sim 10^{-10}$  M, similar to the histidine coordination mode for Cu(II)–A $\beta$ , possibly somewhat larger,<sup>156</sup> with several other amide-involving, concentration-dependent low-affinity sites established as in Cu(II)–A $\beta$ .<sup>210,463</sup>

However, given the weaker binding of Zn(II), it has also been noted that apparent  $K_d$ 's up to  $10^{-6}$  M may strip Zn(II) from amyloids.<sup>228</sup>

In APP, there are both Zn(II) and Cu(II) sites of possible regulatory function with  $K_d$ 's of  $\sim 10^{-6}$  M<sup>288</sup> and  $10^{-8}$  M,<sup>339</sup> again 2 orders of magnitude larger for Cu(II), and close to the solvent-exposed (free) limit and typical for nonactive site, loosely bound, regulatory metal binding sites. Mostly overlooked, these could in fact also be targets for chelators, and some benefits observed with chelators could be due to regulation of APP, not MPAC function, in particular given the weaker binding to APP than to A $\beta$ . Thus, the possibility of selective targeting of either amyloids ( $K_d \approx 10^{-10}$  M for Cu(II)) or regulatory APP metal sites ( $K_d \approx 10^{-8}$  M for Cu(II)) exists, with values for zinc  $\sim 100$  times smaller. Most chelators could also work by lowering the free pools of Zn<sup>2+</sup> and Cu<sup>2+</sup> with  $K_d > 10^{-7}$  M, as MT may do after being secreted by astrocytes and transported through neuronal membrane receptors such as megalin.<sup>464</sup> Therefore, while often discussed in terms of MPAC function, there are in fact at least three modes of function of chelators in AD, depending on their  $K_d$  and cellular localization.

Other prominent examples of chelators include the lipophilic metal chelator DP-109, which reduces amyloid pathology in APP-transgenic mice.<sup>465</sup> Various other Cu–Zn chelators are known to effectively inhibit amyloid formation in AD transgenic mice.<sup>454</sup> The chelator PBT2 has displayed particular potency and is currently in phase II studies and used as a lead in the exploration for new commercial AD drugs.<sup>466</sup> It is important to stress that the molecular causation of these compounds is not clearly established, as they may either resolvent Cu(II)/Zn(II)–A $\beta$  complexes by direct interaction or by noninteracting, competitive binding, or lower free Zn<sup>2+</sup>/Cu<sup>2+</sup>, thus reducing the free metal ion pools, which also affect the amyloid production–clearance balance, as described above.

## 5. OXIDATIVE STRESS AND ALZHEIMER'S DISEASE

### 5.1. Reactive Oxygen and Nitrogen Species

A third hypothesis of AD pathogenesis relates to impaired oxidative stress response in the CNS leading to neurodegeneration.<sup>70–72,467</sup> Oxidative stress is a natural consequence of oxidative phosphorylation within Earth's 21% oxygen atmosphere, and most organisms have evolved to deal with the potential hazards of the ROS that follow in the wake of O<sub>2</sub> metabolism.<sup>468</sup> The most important forms of ROS are outlined in Figure 10, emphasizing their electronic structure and with their spin multiplicity (number of unpaired electrons + 1) given as a superscript before the molecular formula.

Notable ROS are superoxide (O<sub>2</sub><sup>•-</sup>), which is the one-electron-reduced radical anion form of normal triplet dioxygen, dihydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and hydroxyl radical (<sup>•</sup>OH).<sup>469,470</sup> These are formed under all oxidative conditions, but mainly in the mitochondria as a side product of oxidative metabolism. It is estimated that approximately 1–3% of all normal O<sub>2</sub> is converted into ROS in mammals due to inefficiencies of the electron transport chain,<sup>471</sup> and this ROS production can be enhanced if mitochondria are not working optimally due to hypoxic or hyperoxic or other stress-related conditions. ROS that escape the organisms' antioxidant defenses then oxidatively modify nearby proteins and lipids, sometimes rendering them less stable or functional, or nucleic acids of the DNA, leading to mutations.<sup>468</sup>

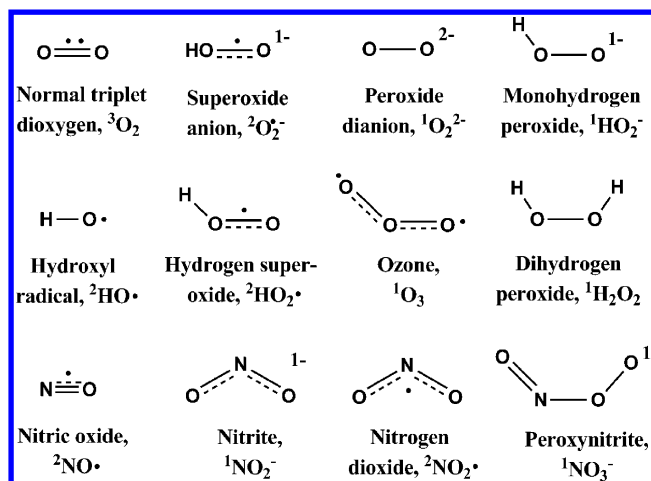
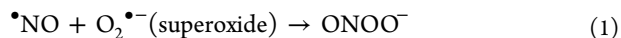


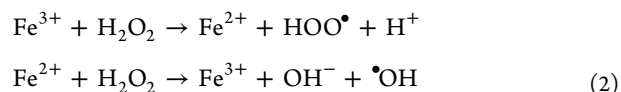
Figure 10. Chemical and electronic structures of normal atmospheric <sup>3</sup>O<sub>2</sub> and reactive oxygen species (ROS, first two rows) and reactive nitrogen species (RNS, last row).

Another notable class of reactive oxidants are the reactive nitrogen species (RNS),<sup>470,472</sup> with the most relevant forms outlined in the last row of Figure 10. These are primarily derived from nitric oxide radical (<sup>•</sup>NO), which is produced by nitric oxide synthase and serves signaling and immune defense roles in the healthy organism,<sup>473</sup> when <sup>•</sup>NO reacts with O<sub>2</sub><sup>•-</sup> to produce peroxynitrite:

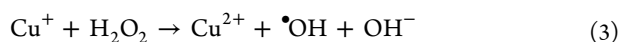


Shifting this reaction to the right, for example, by reduced proficiency of SOD leading to elevated O<sub>2</sub><sup>•-</sup>, causes ONOO<sup>-</sup> to be overproduced. ONOO<sup>-</sup> usually reacts with the plentiful HCO<sub>3</sub><sup>-</sup> to generate carbonate radicals<sup>470</sup> but will also react readily with heme proteins and sulfur and selenium groups of relevance to metal homeostasis and oxidative stress control.<sup>472</sup> ONOO<sup>-</sup> oxidizes cysteines to cystine bridges or oxygenated side chains,<sup>472</sup> and “nitrosative stress” manifests itself, for example, as nitrosylations of protein side chains to impair protein function and stability and deamination of DNA<sup>474</sup> affecting both transcription and mitochondrial metabolism.<sup>475</sup>

Even before considering the vast evidence for metal ion dyshomeostasis and oxidative stress in AD, metal ions play key roles in both ROS production and clearance. Metal ions readily bind ROS and RNS as ligands, and both copper and iron produce hydroxyl radical in solvent-exposed cellular environments, notably via variations of the simplest form of the Fenton reaction:<sup>476,477</sup>



The actual mechanisms can differ substantially<sup>478</sup> but commonly aggravate oxidative stress by converting H<sub>2</sub>O<sub>2</sub> to much more potent hydroxyl. The Fenton chemistry unites metal ion dyshomeostasis, which shifts balance from bound to free metal ions, with oxidative stress pathogenesis, which is predominantly aggravated by free Cu and Fe.<sup>469</sup> The copper redox pair may be involved in similar types of reactions, in particular in the presence of reducing agents such as ascorbate:<sup>479</sup>



Thus, in the absence of direct toxicity of soluble Cu(II)– $A\beta$  oligomers,<sup>190,342</sup> disturbed metal homeostasis resulting in increased concentrations of free intracellular metal ions will itself generate ROS that could lead to oxidative stress. Thus, the two hypotheses are intimately related via a vast number of Cu-, Zn-, and Fe-containing proteins involved in oxidative stress modulation.<sup>480</sup>

### 5.2. The Role of Oxidative Stress in AD

Because of the very large energy need of the brain required to manage the energy-requiring processes of synaptic transmission and ion transport,<sup>481,482</sup> mitochondrial function is particularly sensitive in the brain, and the mitochondrial membranes are central to the regulation of cell death and survival.<sup>70,483</sup> It is also well-established that mitochondrial function declines with age and is correlated with oxidative stress and accumulated gene defects that are particularly abundant in brain, heart, and muscles.<sup>70</sup>

Given that AD is a neurological degenerative disorder that has age as the main risk factor and is characterized by oxidative stress and somewhat relieved by antioxidants,<sup>484,485</sup> it is not surprising that mitochondrial dysfunction and impaired metabolism are early symptoms in AD.<sup>13,71,72,486,487</sup> AD is accompanied by direct structural damage to the mitochondria<sup>488</sup> and reduced glucose utilization.<sup>489,490</sup> Thus, although it is not clear whether mitochondrial dysfunction precedes or follows other pathogenic events, it is central to AD.

Oxidative stress has been suggested to be a primary cause of AD,<sup>491,492</sup> perhaps together with other defining triggers, as claimed in the “two-hit-hypothesis”.<sup>493</sup> Local severe hypoxia, for example, arising from ischemia, leads to oxidative stress because of suboptimal mitochondrial metabolism or damage and plays a significant role in AD.<sup>494,495</sup> Hypoxia leads the mitochondria to produce more ROS, thereby triggering oxidative stress response mediated by the transcription factor HIF,<sup>496</sup> which is not degraded by the iron enzyme prolyl-hydroxylase when either iron or O<sub>2</sub> levels are low.<sup>497</sup> Thus, HIF provides another link between iron dyshomeostasis (section 4.6) and oxidative stress.

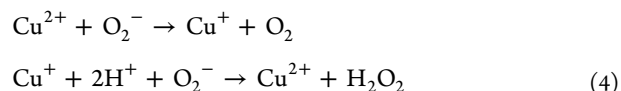
Oxidative stress can however also be caused by the amyloid cascade. As discussed above,  $A\beta$  accumulates and impairs mitochondria,<sup>122,123,125</sup> possibly via apoptosis induced by  $A\beta$  complexes binding to proteins such as alcohol dehydrogenase.<sup>498</sup> Whether  $A\beta$  passes the mitochondrial membrane or is spliced off APP inside the mitochondria is currently unknown.<sup>70</sup> Any toxic effects on mitochondria may itself produce ROS, and the amyloids, at least in the toxic Cu(II) oligomer form, are themselves ROS generators. So even if oxidative stress somehow precedes  $A\beta$  toxicity, both effects are mutually enhancing, creating a detrimental positive feedback<sup>499</sup> that is normally checked by  $A\beta$  clearing.

### 5.3. Links between Oxidative Stress and Other Pathogenic Events

Hypoxia up-regulates  $\beta$ -secretase thereby disturbing the amyloid production-clearance balance and facilitating AD pathogenesis.<sup>88,90</sup> Oxidative stress has also been found to contribute to amyloid production by changing the balance in expression of the three secretase types.<sup>500</sup> The mechanism of this regulation could involve MT and HIF, because both MT-1<sup>501</sup> and MT-3<sup>502</sup> are induced by HIF and the normal, MT-promoting metal-responsive transcription factor-1 (MTF-1).<sup>503</sup> Hypoxia also reduces the uptake and transport of glutamate in astrocytes, which could further facilitate excitotoxicity.<sup>504</sup>

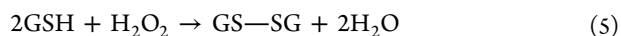
Much more direct evidence for oxidative stress being an underlying cause of the amyloid cascade comes from recent findings of a positive feedback loop between  $\gamma$ - and  $\beta$ -secretase activity triggered by oxidative stress.<sup>88</sup>  $\beta$ -secretase expression is increased by oxidative stress,<sup>505,506</sup> which could explain why hypoxia up-regulates  $\beta$ -secretase, since hypoxia leads to local oxidative stress from metabolic inefficiency, and mitochondrial inhibition has been shown to also up-regulate  $\beta$ -secretase in rats.<sup>507</sup> But  $\gamma$ -secretase activity also increases with oxidative stress and is a cause of the increased  $\beta$ -secretase activity,<sup>88</sup> recently found to be mediated by the produced  $A\beta$ 42.<sup>508</sup> Because oxidative stress naturally correlates with age, accumulated gene errors, and exposure to exogenous risk factors, it explains many risk factors not explained by the amyloid cascade. Also, a positive feedback loop might initiate a sudden, vicious pathogenic cycle upon reaching certain ROS thresholds,<sup>491</sup> potentially explaining the rapid disease progression of AD. Still because metal ions play key roles in the antioxidant system, these findings require a unification.

A central defense against oxidative stress is the SOD-1 and SOD-3 isoforms, which depend on Cu(I)/Cu(II) and Zn(II) in their active sites. While SOD-3 is extracellularly expressed, SOD-1 is located in the intermembrane space of the mitochondria and degrades superoxide that escapes from the mitochondria.<sup>310</sup> Mutations in this enzyme are known to cause ALS<sup>509,510</sup> directly demonstrating the importance of oxidative stress in neurodegenerative disorders. Cu,Zn-SOD catalyzes the two half-reactions



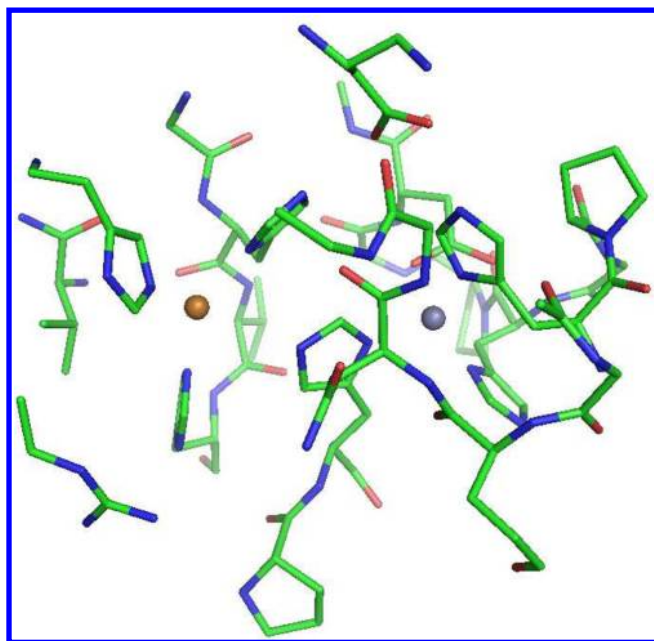
Modification of the SOD-1 active site, either by ~130 mutations identified so far (a cause of familial ALS<sup>511</sup>) or by post-translational modifications, may lead to disrupted metal sites and partial unfolding and a gain in toxic function that is considered a main cause of familial ALS.<sup>273,512,513</sup> The coordination geometry of the active site of extracellular SOD-3<sup>514</sup> is shown in Figure 11. Cu(II)/Cu(I) and Zn(II) are separated by a deprotonated histidine, which may be important in modulating the catalytic cycle of eq 4. Any decrease in metal content, for example, from reduced binding affinity of Zn(II) or Cu(I)/Cu(II), from reduced overall stability of the protein, from stress-induced modifications of the protein that reduce metal affinity, or simply from reduced Zn(II)/Cu(I)/Cu(II) transfer due to functional metal ion deficiency, could impair the function of this enzyme with devastating consequences for the oxidative-stress defenses. Notably, unfolding is correlated with metal loss, so stability/unfolding and metal content mechanisms may occur in concert.<sup>515,516</sup> These ideas are currently pursued in relation to ALS but are relevant also to the neurons involved in AD.<sup>517–519</sup>

Another element that deserves mentioning in the oxidative stress response is selenium, which is abundant in fish and is an ingredient of glutathione peroxidase, an important antioxidant enzyme that converts H<sub>2</sub>O<sub>2</sub> to water via GSH, eq 5:



Selenium may also detoxify heavy metals such as Hg by direct binding and excretion from human tissue,<sup>520</sup> but as recently reviewed, there are no clear indications of a beneficial role of selenium in AD.<sup>521</sup>





**Figure 11.** Extracellular Cu,Zn-superoxide dismutase (SOD-3) (2JLP.pdb at 1.7 Å resolution). Cu (orange) is to the left, whereas Zn (blue) is to the right. Between them is a bridging histidine imidazololate. See ref 514 for details.

$\text{H}_2\text{O}_2$  can also be reduced by various peroxidases in addition to MT, SOD, and GSH, and several of these depend directly on the available heme iron pool, because both some peroxidases and some catalases are heme enzymes.<sup>522,523</sup> Disruption of the balance between the free and bound iron pools may thus contribute to oxidative stress imbalance both by increased production of ROS and by reduced degradation of ROS. ROS in themselves, in particular superoxide  $\text{O}_2^{\bullet-}$ , also affect neuronal signaling by activation of NMDA-R, and enhanced expression of SOD reduces glutamate sensitivity, directly linking metal-dependent ROS scavenging to glutamate signaling.<sup>70,524</sup>

In conclusion, oxidative stress pathogenesis correlates with metal ion dyshomeostasis where the bound metal-ion pool protects against ROS/RNS via antioxidant enzymes, whereas the free metal-ion pool produces them via Fenton chemistry. Thus, these two hypotheses can be united with causal relations among various pathogenic events, and metal ion dyshomeostasis, the shift from bound to free metal ion pools, can explain many features of oxidative stress associated with neurodegeneration.<sup>377</sup>

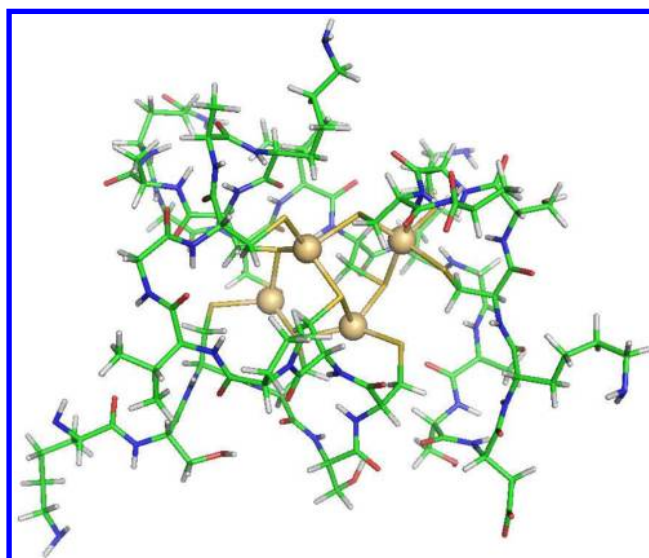
## 6. METALLOTHIONEINS AND ALZHEIMER'S DISEASE

### 6.1. Structure, Expression, and Roles of Metallothioneins

If a protein is implicated in dyshomeostasis, it can be due to changed production or clearance or protein modification, either post-translationally or due to mutations. To understand causative factors of zinc dyshomeostasis in neurological disorders, three classes of proteins are particularly relevant:<sup>10</sup> metallothioneins (MT),<sup>525</sup> ZIP transporters,<sup>281</sup> and Zn transporters (ZnT),<sup>164,526</sup> the latter transporting  $\text{Zn}^{2+}$  across membranes to the cytoplasm. In ZnT3-knockout mice, free  $\text{Zn}^{2+}$  accumulates from intracellular stores not associated with the synaptic vesicles, pointing toward MTs as central to the buffering of bound and free zinc pools.<sup>297</sup>

MTs<sup>527,528</sup> are small (60–68 residues) cysteine-rich proteins that bind Cd(II), Cu(I), and Zn(II).<sup>529–531</sup> The known isoforms of mammalian MTs have seven divalent metal ions each bound to four cysteine sulfur ligands, whereas monovalent metal ions such as Cu(I) may bind in higher numbers than 7, with lower coordination numbers of 2 or 3.<sup>530,531</sup> Cd(II) and Zn(II) are symmetric  $d^{10}$  ions giving formally  $T_d$  coordination geometry, but the protein renders all metal sites inequivalent, imposing different metal affinities to each metal site.<sup>532</sup>

In mammals, four main MT classes are distinguished, with MT-1 and MT-2 present in most tissue, whereas MT-3 and MT-4 appear mainly (but not exclusively) in specialized tissue such as the brain and skin.<sup>533</sup> Brain MT-1/MT-2 is mainly expressed in glia cells and mainly in the astrocytes<sup>534</sup> that maintain homeostasis in the CNS, the integrity of the BBB, and the metabolism of neurons,<sup>464</sup> express extracellular metalloproteases that degrade amyloids,<sup>94</sup> and accumulate toxic exogenous metals such as Pb (possibly in MTs).<sup>316</sup> MT-3 is particularly abundant in ZEN and astrocytes<sup>535</sup> in the cerebellar cortex, and in particular in the hippocampus degenerated early in AD,<sup>4,8</sup> in amygdala, and in the olfactory bulb.<sup>10,536</sup> In human MT-1 and MT-2 (see structure<sup>537</sup> in Figure 12), Zn(II) is the



**Figure 12.** Structure of the  $\alpha$ -domain of human MT-2 with four Cd(II) ions bound (structure 1MHU.pdb, ref 537.).

dominating metal bound, whereas both Cu(I) and Zn(II) are found in MT-3.<sup>538</sup> There are more than 10 isoforms of MT-1, two of MT-2 (MT-2a and MT-2b), and one of MT-3/MT-4.<sup>539</sup>

MT-1 and MT-2 are highly sequence-similar, whereas MT-3 is the only MT isoform with a negative total charge, due to its acidic insert. MT-3 is thus substantially negatively charged, while other MT isoforms are nearly neutral. In mammalian cells, most MTs appear to be N-acetylated at the N-terminus, reducing the charge further.<sup>540</sup>

### 6.2. Specific Functions of Metallothioneins

MTs are responsible for transport, storage, and regulation of zinc and copper,<sup>541,542</sup> detoxification of heavy metals<sup>543,544</sup> (most Cd(II) is bound to MT *in vivo*),<sup>545</sup> antistress functions,<sup>546</sup> including in particular oxidative stress,<sup>539,547</sup> anti-inflammation<sup>548</sup> and cell regeneration,<sup>549,550</sup> and anti-apoptosis.<sup>539</sup> MT transcription is substantially (>5-fold) up-regulated by free  $\text{Zn}^{2+}$ , stress-induced interleukins,<sup>539,551</sup>

glucocorticoid,<sup>541,552,553</sup> epinephrine and norepinephrine,<sup>554</sup> heavy metals such as Cd(II), or ROS.<sup>527,555</sup> MT induction occurs by Zn<sup>2+</sup> binding to the zinc-finger transcription factor MTF-1.<sup>556,557</sup> All MT genes contain several metal-responsive elements (MRE) in their promoter regions.<sup>555</sup>

The loss of Zn(II) from MT most likely occurs upon heavy metal substitution or thiolate oxygenation and subsequent cystine bridge formation by ROS.<sup>539</sup> In the harbor porpoise, large fractions of the total Cd, Cu, and Zn, but not Hg, were bound to MTs,<sup>558</sup> indicating that Cd toxicity, but not Hg toxicity, is countered by MTs. The role of MT in controlling zinc homeostasis is seen in the fact that it prevents both zinc deficiency and toxicity.<sup>559</sup>

Thionein (T), the apoprotein form of MT, removes Zn<sup>2+</sup> from a wide range of inhibitory sites, thus activating a number of enzymes selectively without impairing enzymes that use Zn(II) in active sites<sup>560,561</sup> or zinc-fingers.<sup>562,563</sup> On the other hand, Zn-MT is known to transfer Zn(II) to a variety of enzymes,<sup>564</sup> for example, carbonic anhydrase,<sup>565</sup> and to zinc-fingers of, for example, estrogen receptors,<sup>566</sup> indicating that MT is a central regulator of Zn availability in zinc-dependent proteins.<sup>567,568</sup> This transfer can be redox-dependent, for example, via coupling to the glutathione GSH/GSSG redox couple.<sup>561</sup>

The redox potential of MT of approximately  $-366$  mV means that upon oxidation of cysteines, MT can release zinc to transcription factors involved in oxidative stress defense, and thus function as a central redox sensor of the brain.<sup>10</sup> This process can be enhanced by glutathione disulfide (GSSG).<sup>569,570</sup> MT substantially reduces oxidative stress in rats and mice.<sup>571–574</sup>

### 6.3. Investigated Roles of Metallothioneins in AD

Due to the protective role of MT against apoptosis,<sup>575,576</sup> its role in zinc- and copper-homeostasis<sup>327</sup> and the central nervous system,<sup>464,577,578</sup> and the abnormal MT expression in AD brains,<sup>579,580</sup> MT is now appreciated as playing a significant, yet poorly understood role in AD.<sup>581–583</sup> MT also plays major roles in other zinc-related disorders such as diabetes,<sup>584</sup> ALS,<sup>273</sup> autism,<sup>271</sup> epilepsy,<sup>585</sup> and multiple sclerosis<sup>586</sup> and in a variety of aging processes,<sup>587</sup> where MT is among very few proteins that correlate positively with life longevity in mice.<sup>588</sup> Absence of MT reduces cognitive function in mice<sup>589</sup> and renders them less resistant to induced seizures.<sup>590</sup>

MT-3 levels are generally reported to be changed in patients with AD, but the changes are similar to those for zinc with regional redistributions and diverging reports,<sup>579,580,591</sup> whereas MT-1 and MT-2 are generally up-regulated.<sup>592,593</sup> MT-1 and MT-2 up-regulation in AD and other neurological disorders may be a host defense response reflecting the pathology and inflammatory signals, which induce MT via glucocorticoids or elevated free intracellular Zn<sup>2+</sup> binding to MTF-1.<sup>527</sup> However, Zn-MT-1/MT-2 levels in the liver are reduced despite this up-regulation,<sup>594</sup> suggesting a pool of (possibly modified) apothionein or MT bound to other metals than Zn(II).

MT-3 can limit cell death and enhance neurite (axon/dendrite) growth by identified signaling pathways<sup>595,596</sup> and protects neurons from cerebral ischemia in mice.<sup>597</sup> The protein is, like MT-1/MT-2, released from housekeeping astrocytes<sup>428,429</sup> during brain injury and blocks axon regeneration while it eliminates ROS.<sup>598</sup> Matrix metalloproteases that degrade extracellular amyloids and require Zn(II) are also expressed by astrocytes.<sup>94</sup> Once the oxidative stress is

eliminated, MT-3 is down-regulated so that neuronal regeneration can proceed.<sup>583</sup> Thus, MT-3 has a periodic response to neuronal insult that involves both cell clearance and regeneration, whereas other isoforms are uniformly protective.<sup>464,591</sup> MT-3 can bind more metal ions and exhibits more dynamic mixtures of metalloforms, that is, its role in Zn-buffering and sensor/signaling in ZEN is plausibly different from other MT isoforms.<sup>599</sup>

Reactive astrocytes with high expressions of MT are found in AD patients (*astrogliosis*), and the correlation is significant enough to suggest MT levels as a marker of AD.<sup>600</sup> MT-1/MT-2 is released to extracellular space by astrocytes<sup>601</sup> to promote neuronal regeneration via signal transducer and activator of transcription 3 (STAT3),<sup>602</sup> a central transcription factor that activates a number of genes involved in cell growth, differentiation, and apoptosis. Together with MT-1/MT-2, other protective extracellular proteins such as SOD-3 and prion protein are also released from astrocytes during neuron damage.<sup>464</sup> Extracellular MT-1/MT-2 is internalized by nearby neurons via megalin receptors on the neuronal cell surface.<sup>464</sup>

Given their buffering roles and protecting role in scavenging Cu(II)-A $\beta$ ,<sup>247</sup> MTs may constitute a “gold standard” for the rational design of new MPACs. Both MT-3<sup>603</sup> and MT-2<sup>604</sup> protect neurons from the toxicity of A $\beta$  peptides, and MT-3 can reduce neurodegeneration in mouse hippocampus.<sup>605</sup> Most importantly, it was recently shown that Zn-MT-3 extracts Cu(II) from the Cu-A $\beta$ 40 complexes in exchange for Zn(II),<sup>247</sup> and MT-3 interaction renders the amyloids nontoxic, indicating that the toxic amyloid oligomers or precursors to the toxic oligomers contain Cu(II) or Zn(II).<sup>606</sup> Recently, a similar ability to prevent A $\beta$  oligomer formation was confirmed for MT-2,<sup>607</sup> although studies of MT-2 and MT-3 binding to the protein transthyretin and A $\beta$  suggest that MT-2 may not affect amyloids in the same way as MT-3.<sup>608</sup> MT-2 injected into mice subject to AD-like pathology (Tg2576-type) improves cognition but also increases plaque load.<sup>582</sup>

As discussed in section 4.2, the  $K_d$ 's for Cu(II) and Zn(II) binding to A $\beta$  lie typically around  $10^{-10}$ – $10^{-9}$  M and  $\sim 10^{-7}$  M, respectively.<sup>192,609</sup> The regulatory Cu<sup>2+</sup> and Zn<sup>2+</sup> sites of APP display  $K_d$ 's of  $\sim 10^{-8}$  M<sup>339</sup> and  $\sim 10^{-6}$  M,<sup>288</sup> that is, 1 order of magnitude weaker binding, and the Zn<sup>2+</sup> site regulates  $\alpha$ -secretase activity.<sup>288</sup> As mentioned, Cu(II) typically binds more strongly than Zn(II) to most chelators due to the Irving–Williams series.<sup>250</sup> For comparison, active-site Zn(II) in zinc enzymes such as carbonic anhydrase<sup>610</sup> and Cu,Zn-SOD<sup>611</sup> is more strongly bound, with typical  $K_d$ 's of  $\sim 10^{-11}$  M.<sup>568</sup> An effective chelator against metal-amyloid toxicity should not outcompete these sites, suggesting an MPAC in a very narrow range between bound and free pools with a  $K_d$  of  $\sim 10^{-10}$  M for Cu(II) and perhaps  $10^{-8}$  M for Zn(II). Apo-MT-1/2 displays  $K_d$ 's for Zn(II) on the order of  $10^{-13}$  M<sup>567,612</sup> but is still the main Zn(II) buffer within eukaryotic cells,<sup>613</sup> because Zn(II) transfer occurs stepwise from partially metalated forms, notably Zn<sub>5</sub>-MT and Zn<sub>6</sub>-MT with  $K_d$ 's of  $\sim 10^{-10}$  M,<sup>614</sup> very close to the optimal range suggested above. Zn<sub>7</sub>-MT-1/2 has been found to have one weakly bound Zn(II) with  $K_d \approx 10^{-8}$  M and donates this Zn(II) to other chelators.<sup>614</sup> For MT-3, an additional eighth Zn(II) binding site has been identified that is not present in MT-2.<sup>615</sup>

For both MT-2 and MT-3, stronger binding of Cu(II) as expected from the Irving–Williams series has been confirmed by density functional calculations.<sup>616</sup> It would be desirable to

design MPACs selectively targeting each metal ion, to prevent dangerous stripping of active site metal ions,<sup>192</sup> and MT could be a central model for such designs. The natural chelator function of MTs also applies to MT-3, which scavenges oxidative-stress-generated Cu(II).<sup>617</sup>

The degradation of MTs by cysteine proteases such as cathepsin B occurs actively in lysosomes,<sup>618,619</sup> and by certain other proteases in the cytosol.<sup>539,620</sup> This is interesting because cathepsin B has also been shown to have a neuroprotective effect, plausibly by degradation of A $\beta$ .<sup>621</sup> Thionin is more prone to degradation, suggesting that clearance is enhanced if metal sites are modified to reduce the binding affinity. This could explain why the Cu/MT ratio may be a reliable marker of AD progression,<sup>622</sup> that is, the free Cu<sup>2+</sup> pool increases more than the MT levels and is correlated in time with AD progression.

## 7. METABOLISM, AGING, DIABETES, AND ALZHEIMER'S DISEASE

### 7.1. Metabolism, Aging, and AD

The risk factors of hypercholesterolemia, obesity, and diabetes, and the benefits of exercise and mental activity associated with AD may reflect that imbalances in mitochondrial metabolism are early pathogenic events.<sup>13,623</sup> As advocated by the free-radical theory of aging,<sup>468,624,625</sup> slower metabolic rate, for example, induced by moderate hypoxia,<sup>626</sup> enhances life span by producing less radical oxidative damage from mitochondrial activity, whereas hyperoxic conditions shorten life span of cultured cells.<sup>627</sup> Evidence for the association between mitochondrial ROS and aging was obtained from longevity observed in mice overexpressing mitochondrial catalase.<sup>628</sup> It is important to distinguish local severe hypoxia, for example, from ischemia, and moderate hypoxia, for example, that associated with living at high altitude. The latter will contribute to the effect of controlled, slower metabolism, whereas the former generates mitochondrial damage and ROS.<sup>496</sup>

During aging, a number of significant changes occur that are related to changing gene regulation and protein expression.<sup>629</sup> Some of these normal aging processes converge with symptoms of AD: (1) genes that enable synaptic function, for example, NMDA-R, GABA-R, and voltage-gated Na channels, are down-regulated;<sup>629</sup> (2) Ca<sup>2+</sup> levels are elevated in older people<sup>630</sup> and calcium homeostasis is down-regulated<sup>364,629</sup> in proportion to down-regulated synaptic transmission; (3) inflammation and stress responses, for example, MT-1/MT-2, integrin, and heat shock proteins, are up-regulated;<sup>629</sup> (4) mitochondrial function is impaired and down-regulated.<sup>629</sup> Hormonal changes are diverse: leptin and insulin receptors are up-regulated, whereas melatonin, controlling the circadian rhythm (*vide infra*), and somatostatin, involved in neurotransmission, are down-regulated, consistent with less synaptic activity.<sup>629</sup>

Consequently, in many ways, the AD brain is a brain that has aged faster than the average brain,<sup>364</sup> and one could in some aspects view AD as an “accelerated-aging” disorder.<sup>631</sup> This is supported by the broad clinical spectrum of the disease, as well as the occurrence of amyloid plaques in people without AD.<sup>631</sup> AD patients also display reduced glucose uptake, which is one of the common biomarkers of AD.<sup>6</sup> AD is currently often diagnosed via memory impairment confirmed by presence of senile plaques during post-mortem autopsy, with biomarkers playing a rather small role.<sup>6</sup> Differences between AD brains and accelerated aging brains should be obtainable from comparing

AD brains to older brains instead of same-age controls and would be relevant to define how AD distinguishes itself from the accelerated aging brain, and not just the same-age control brain. For example, the expression of metalloproteins such as MT-3 are elevated in elder but are abnormally distributed in AD,<sup>535</sup> which is clearly not simply an accelerated aging effect but possibly a result of redistributed zinc levels and astrocyte dysfunction (*vide infra*).

In the aging body, the mitochondrial down-regulation correlates with less physical and mental activity (synaptic function is also down-regulated) and is associated with oxidative DNA damage consistent with the free radical theory of aging.<sup>629</sup> One of the most sensitive mitochondrial proteins subject to oxidative damage is mitochondrial aconitase, an iron–sulfur containing enzyme in the Krebs cycle that converts citrate to isocitrate, which is impaired by superoxide.<sup>632</sup> Thus, one of the first critical imbalances in oxidative stress-induced aging may be associated with aconitase.<sup>632</sup> This is interesting since MT transfers Zn(II) to mitochondrial aconitase in mouse hearts and may thus regulate the Krebs' cycle,<sup>633</sup> as also indicated by the effect of zinc on the isocitrate/citrate equilibrium of aconitase.<sup>634</sup> Free Zn<sup>2+</sup>, which would arise from dysfunctional MT, has instead been found to inhibit cytochrome *c* oxidase<sup>334,635–637</sup> and mitochondrial aconitase synthesis,<sup>638</sup> thus reducing metabolism and energy production.

Another important marker of aging is the shortening of telomeres, the DNA sequences that protect the ends of chromosomes from damage.<sup>639</sup> The reverse transcriptase telomerase adds new protecting DNA sequences to telomeres of eukaryotic stem cells and some cancer cells, but not to other cells, which are then subject to a “count-down” of telomere shortening upon replication that may eventually result in chromosome fusion.<sup>640</sup> The activity of telomerase, as a reverse transcriptase, is up-regulated by Zn<sup>2+</sup>, thus preserving the ability to replicate<sup>641</sup> and in some sense “slowing the count-down”, whereas artificial Zn-finger motifs can repress telomerase.<sup>642</sup> A casual link between zinc homeostasis and telomere shortening, including an inverse relationship between MT expression and telomere shortening in people older than 80 years has recently been indicated.<sup>643</sup> Thus, zinc homeostasis may be centrally positioned within the biological clockwork of aging, with the free Zn<sup>2+</sup> pool at least to some extent directing the speed of the clock.

### 7.2. Zinc: A Link between Diabetes and AD?

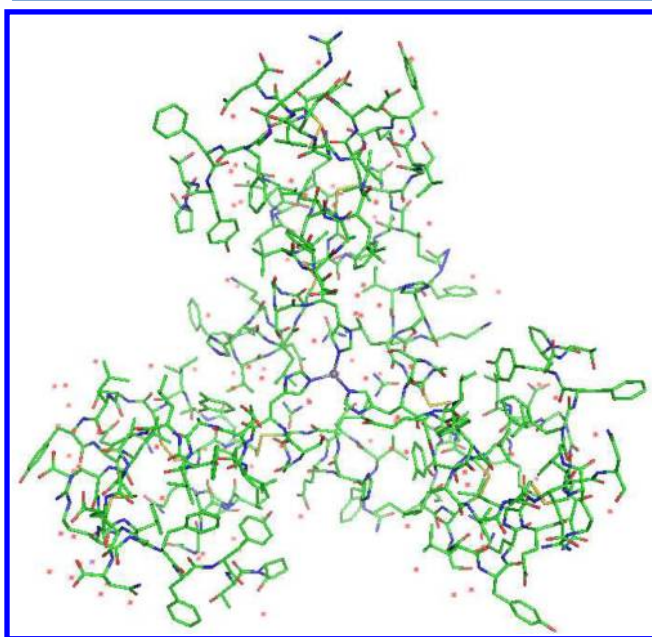
While being completely different diseases in terms of pathology and progression, there are several underlying similarities between AD and type II diabetes:<sup>644</sup> AD brains are insulin-resistant,<sup>384,645</sup> and such insulin resistance leads to cognitive decline;<sup>646</sup> both diseases are accompanied by oxidative stress and have age as a risk factor;<sup>647</sup> diabetes is itself a risk factor for AD,<sup>20,21</sup> as is obesity and hypercholesterolemia,<sup>10</sup> and diabetes aggravates AD symptoms.<sup>648</sup> The similarities between protein aggregations in the two diseases were recently reviewed.<sup>649</sup>

A common feature of the diseases is zinc dyshomeostasis, widely documented in both type-I and type-II diabetes.<sup>647,650–653</sup> Increased urinary excretion of zinc is observed in diabetes patients suggesting elevated free Zn<sup>2+</sup> as in AD.<sup>654,655</sup> On the other hand, type-I and type-II diabetes are usually associated with higher vs lower blood zinc levels, respectively.<sup>650,656,657</sup> Zinc can partly remedy type-II diabetes by showing insulinomimetic activity,<sup>658</sup> but while its role in insulin signaling and integrity, storage, and transport is



established,<sup>651,659</sup> the mechanism of this function is not understood.<sup>650</sup>

Zn<sup>2+</sup> is required for the stable storage of insulin, because it binds in the center of the insulin hexamer complex and is tightly correlated to insulin storage and release,<sup>650,660</sup> see Figure 13.<sup>661</sup> Zinc dyshomeostasis changing the equilibrium between



**Figure 13.** Structure of one half of the human insulin hexamer stabilized by tetrahedrally coordinated Zn<sup>2+</sup> in the center (2OMG.pdb). See ref 661 for details.

free and bound zinc pools might disrupt this structural integrity, possibly explaining the reduced glucose utilization in AD. Furthermore, the insulin degrading enzyme is also an important Zn(II)-dependent amyloid-degrading protease (Figure 7).<sup>292</sup>

These suggestions are supported by the observation that MT-knockout mice experience increased zinc loss via the pancreas,<sup>662</sup> showing that dysfunctional MTs may contribute to zinc dyshomeostasis also in diabetes. The beneficial effect of MT on type-2 diabetes is well established,<sup>650,663</sup> and MT is of central importance in preventing diabetic cardiovascular complications,<sup>664–666</sup> diabetes-related oxidative stress,<sup>667</sup> diabetes-related leptin imbalance, and obesity.<sup>668,669</sup> Certain MT polymorphisms are correlated with type-2 diabetes.<sup>670</sup> Zn-induced MT remedies induced diabetes by protecting against oxidative stress,<sup>572,573</sup> and MT function has been observed to be impaired in type-2 diabetes.<sup>671</sup> Reconstituted zinc transfer to relevant targets, including insulin, SOD, or transcription factors, are plausible explanations for these benefits,<sup>672,673</sup> although zinc alone also has a beneficial effect uncorrelated with the MT that zinc induces.<sup>573</sup>

Since ROS cause insulin resistance,<sup>674,675</sup> antioxidative dysfunction is a possible focal point of common pathogenic mechanisms in AD and diabetes, and MT's role in type-2 diabetes could also relate to its antioxidant function, which is intimately related to its zinc binding.<sup>676</sup> However, another link is iron dyshomeostasis, which disrupts the efficiency of the mitochondria's energy production and could both affect glucose utilization and insulin balance and cause ROS via Fenton chemistry.<sup>161,677</sup> Mutations in ceruloplasmin, the multicopper ferroxidase important in iron homeostasis described in section

4.8, have been reported to cause both diabetes and neurodegeneration.<sup>424</sup>

The links between diabetes and AD are currently mainly hypothetical and should not be overemphasized, but the common pathological features could in principle be explained mechanistically by zinc dyshomeostasis, and this hypothesis warrants further investigation.

## 8. METHIONINE SYNTHASE, VITAMIN B<sub>12</sub>, AND HOMOCYSTEINE IN ALZHEIMER'S DISEASE

As described above, hyperhomocysteinemia (~15–50 μmol/L)<sup>678</sup> is correlated with risk of AD<sup>22,24,679</sup> although there are diverging reports as recently reviewed.<sup>680</sup> Homocysteine levels are also elevated in cerebral ischemia,<sup>681</sup> cardiovascular disease (together with Cu),<sup>682</sup> and various other disorders.<sup>683–685</sup> In humans, homocysteine is converted to methionine by the enzyme methionine synthase (MES),<sup>686</sup> which plays a central role together with S-adenosyl methionine (SAM) in methylation pathways and in nucleotide synthesis in the CNS.<sup>678,687</sup>

MES both depends on the cobalt-containing cobalamin cofactor for transferring methyl in an S<sub>N</sub>2 reaction and requires Zn(II)<sup>688,689</sup> as do other methyl-transfer enzymes,<sup>690</sup> most likely to enhance the nucleophilicity of homocysteine by lowering the pK<sub>A</sub> and deprotonating the sulfur-bound proton before nucleophilic attack on cobalamin-bound methyl.<sup>691</sup> Thus, MES dysfunction due to decreased availability of Zn(II) or vitamin B<sub>12</sub> (including cobalt) or zinc-mimetic inhibition, for example, by Hg,<sup>692</sup> will lead to hyperhomocysteinemia. The physiological effects of hyperhomocysteinemia and vitamin B<sub>12</sub> have been reviewed recently,<sup>678,693</sup> and some of the most repeatedly observed effects are elevated Ca<sup>2+</sup> levels, oxidative stress, cell death/caspase-3 activation, and higher intracellular Aβ<sub>42</sub>.

The underlying cause of the hyperhomocysteinemia observed in AD can be due to either production or clearance issues. In terms of clearance, Zn(II)-deficient MES could lead to homocysteine buildup, possibly explaining why B<sub>12</sub> or folic acid supplements do not slow AD progression,<sup>694,695</sup> although this could also be because the homocysteine balance is quite downstream from the causative events in AD. Also, colocalization of exogenous homocysteine with amyloids<sup>696</sup> could indicate that homocysteine is not cleared because it is bound to Zn(II) and Cu(II) in extracellular amyloid deposits, although this remains to be investigated further.

Importantly, the methylation pathways controlled by MES and SAM also underlie the conversion of norepinephrine to adrenaline by phenylethanolamine N-methyltransferase, which has been found to be down-regulated in AD,<sup>697</sup> plausibly because of the homocysteine buildup upstream to this enzyme. Thus, the production of norepinephrine (and hence adrenaline) is impaired in AD, and the hormone has been found to enhance phagocytosis of Aβ,<sup>698</sup> which could aggravate AD. An association between some variations in phenylethanolamine N-methyltransferase and AD has been documented.<sup>699</sup> As mentioned previously, a part of the MT stress response is due to induction of MT by epinephrine and norepinephrine,<sup>554</sup> and if this induction is absent, it may enhance inflammation in neurons usually reduced by MT.

## 9. ALS AND AD: SAME THING, BUT DIFFERENT

ALS and AD share a variety of pathological features:<sup>273,512,513</sup> oxidative stress, neuronal inflammation, dysfunctional zinc

homeostasis, mitochondrial malfunction, formation of protein aggregates (amyloids in AD; neurofilaments in ALS), only a few years survival time after diagnosis, late onset (i.e., age risk), substantial increase in occurrences since the 1960s or 1970s indicating some environmental risk factors that cannot be fully explained by changes in diagnostic paradigms,<sup>700</sup> and symptoms of apoptosis signaling in the disease end stage.<sup>273,701</sup> Both disorders have a minority of familial cases (in ALS due to SOD-1 mutations causing ~20% of familial cases<sup>701</sup>), while the vast majority of cases are sporadic. Motor neurons are, like neurons in the brain, consumers of large amounts of energy, rendering their mitochondria more sensitive to oxidative stress.

Several research groups have established that a fundamental cause of the fraction of familial ALS relating to SOD-1 mutations is a gain of toxic function of SOD-1,<sup>702–704</sup> and such toxic function, for example, aggregation or partial unfolding, is linked to reduced Zn(II) affinity,<sup>705</sup> suggesting lack of Zn(II) in the protein. Some argue for<sup>706,707</sup> or against<sup>708</sup> correlation between disease progression and protein stability. As described recently,<sup>273</sup> lack of Zn(II) in SOD-1 could reverse the reaction so as to produce superoxide radicals from O<sub>2</sub>, giving nitrosylated proteins and lipids,<sup>709</sup> but other toxic functions are possible, for example, partial unfolding and aggregation coupled to new redox chemistry, such as, exposure of copper leading to Fenton-type oxidations, eq 3. Also,  $\beta$ -secretase overexpression can reduce SOD-1 activity,<sup>710</sup> possibly by protein–protein interactions mediated by Cu,<sup>69</sup> providing a possible molecular mechanistic link between AD and ALS pathogenesis.

In contrast, sporadic ALS seems to be caused by gradually enhanced stress leading either to steady progression of the disease or to sudden pathogenic cascades due to key events relating primarily to oxidative stress.<sup>491</sup> Given the similarity in sporadic and familial ALS pathogenesis,<sup>711</sup> it seems logical to assume that SOD-1 dysfunction is also a plausible cause of sporadic ALS and familial ALS not directly related to SOD-1 mutations. Dysfunctional SOD-1 would then be caused by post-translational modifications, for example, lack of Zn(II) for other reasons than mutations decreasing Zn(II) binding affinity. Given MT's role in buffering Zn(II), it is a plausible hypothesis that dysfunctional MT is a cause of dysfunctional SOD-1 in sporadic ALS, a hypothesis that has indeed been supported.<sup>701,712</sup> This would put AD and ALS into close pathogenic relationship, although the cellular locations (motor neurons vs zinc-enriched neurons in hippocampus,<sup>10</sup> possibly both due to astrocyte dysfunction) and thus neurological consequences of the diseases would differ. Alzheimer-type tau pathology has also been associated with SOD-1 deficiency, and SOD-1 was found to be reduced in AD patients compared with other mitochondrial and extracellular SODs.<sup>713</sup>

Mouse models of accelerated aging (SAMP10) display zinc deficiency due to low expression of ZnT3.<sup>714</sup> Very recently it was found that MT-3 significantly prolongs life span of ALS model mice and reduces motor neuron loss.<sup>715</sup> MT levels are elevated in kidney and liver of ALS patients,<sup>716</sup> and MT expression is increased in the spinal cord of ALS patients.<sup>717</sup> A variety of heavy-metal exposures have been found to lead to ALS<sup>718</sup> or ALS-like symptoms.<sup>719</sup> However, while zinc deficiency was linked to ALS in a 22-person study, heavy metals in toe nails did not correlate with ALS.<sup>720</sup> Larger studies and consideration of other heavy metal markers (e.g., MT

markers where most Cd is located *in vivo*<sup>545</sup>) instead of toe nails would be warranted.

As in AD, in the final disease stage of ALS, when motor neuron mitochondrial damage reaches a certain critical level and oxidative stress is no longer sustainable, apoptosis may be triggered by release of cytochrome *c* and apoptosis-inducing factor (AIF) from mitochondria, initiating the caspase cascade,<sup>721</sup> which is also regulated by free Zn<sup>2+</sup>.<sup>722</sup>

Of further interest is the possibility of a similar mode of toxicity in the mutant SOD-1 of familial ALS and of Cu(II)/Cu(I)–A $\beta$ , notably given the similarities between the plausibly partly unfolded and hence solvent-exposed SOD-1 Cu(II)/Cu(I) site and the corresponding site in amyloids,<sup>723</sup> sharing doubly coordinated histidine and potentially forming ROS-generating exposed Cu(I)/Cu(II) Fenton chemistry. As discussed, in addition to its ferroxidase function, APP contains both copper and zinc binding sites with potential redox function that might be linked to amyloid processing (e.g., a ferroxidase/SOD metabolic control function of mitochondria). The hypothesis that the key toxic mode of A $\beta$  is a solvent-exposed Cu(II)-motif chemically and structurally similar to the acquired toxic function of SOD-1 mutants would be interesting to investigate.

## 10. EXOGENOUS METAL EXPOSURE AND ALZHEIMER'S DISEASE

Since the early 20th century and in particular since 1950, the industrial production and emissions of exogenous metals have dramatically increased.<sup>724</sup> This enhances the risk of exposure to the CNS, both during development and in adults. The neurotoxic effects of non-natural metals, which are well-established,<sup>725–728</sup> depend on the scale and duration of the exposure and how it coincides with the delicately tuned developmental processes of the CNS.<sup>729</sup> Notably, many neurological disorders, including AD and ALS, have been subject to accelerating prevalence in postwar history that could be partly due to increased awareness and partly due to exogenous factors such as urban pollution.

### 10.1. Aluminum

The first exogenous metal to be identified as a risk factor in AD was aluminum (Al).<sup>730</sup> Aluminum is a light metal (atomic number 13) with iron-mimetic biochemical behavior.<sup>44,731</sup> While many toxic mechanisms of Al are known, none of these have been causally linked to AD.<sup>45</sup> In the 1990s, several research groups found no elevated Al levels in AD tissue,<sup>732–734</sup> and there were disagreeing *in vivo* and *in vitro* studies<sup>735,736</sup> resembling somewhat those for Zn, Cu, and Fe, with highly tissue-dependent and heterogeneous reports. Furthermore, Al is often easily excreted<sup>737</sup> and did not seem to penetrate to the brain in toxic concentrations that could justify an “aluminum hypothesis” of AD.<sup>738</sup> Also, some reports of elevated Al were shown to be erroneous.<sup>732</sup> However, after substantial controversy,<sup>739,740</sup> Al is now mostly considered a risk factor in AD, as reviewed recently by several authors,<sup>44–46,741</sup> although its total weight among other risk factors is highly debatable.<sup>147,742</sup>

Approximately 20 studies have related Al in diet and drinking water to AD risk<sup>44,730,743–749</sup> (more references can be found elsewhere<sup>743</sup>). Still, most Al exposure occurs via inhalation in Al-involving industries.<sup>725</sup> Early or midlife exposure to Al has been found to be a significant cause of later AD development among foundry workers.<sup>750</sup> In dialysis patients, Al accumulates

in the brain and gives rise to a form of neurodegeneration called dialysis encephalopathy,<sup>751</sup> with symptoms that resemble AD.<sup>725,752</sup> In AD brains, while Al levels were not found to be toxic, as with other metal ions, there are large heterogeneities in the local depositing of the metal, typically leading to local toxic concentrations.<sup>153</sup>

The detailed toxicological aspects of aluminum are reviewed elsewhere,<sup>725</sup> but for the present context, it is most relevant to note that since Al(III) is a hard Lewis acid that mimics Fe(III), its primary toxicological targets are different from the heavy metals discussed below. Al(III) mainly interacts with the mitochondria and impairs energy production, for example, by interference with iron–sulfur proteins of the respiratory system.<sup>753</sup> Recent studies provide possible mechanisms for Al's involvement in amyloid formation *in vitro*<sup>178,252,754</sup> and *in vivo*,<sup>44,755</sup> and in AD-like tau processing.<sup>756,757</sup> Aluminum exposure was recently shown to produce iron-mediated ROS in *Drosophila* of relevance to AD,<sup>758</sup> and the ability of iron chelators to remedy this ROS<sup>758</sup> suggests that Al(III) has substituted bound Fe(II/III), probably leading to free Fe<sup>2+</sup>/Fe<sup>3+</sup> and Fenton toxicity, which would enhance the ROS burden and render the CNS more vulnerable to AD risk factors. A scheme for aluminum's interference with iron regulatory proteins leading to increased risk of AD have been discussed in several reviews.<sup>144,383</sup>

As other metal ions, Al(III) also binds directly to A $\beta$ ,<sup>240</sup> is colocalized in senile plaques,<sup>759</sup> and may in fact produce structural aggregates that are more toxic than those with other metal ions, while also interrupting tau protein processing.<sup>760</sup> The aggregation mechanisms of Al(III)–A $\beta$  were also found to be distinct from those of Cu(II) and Zn(II).<sup>240</sup> In fact, it has independently been suggested that trivalent metal ions such as Al(III) are key to the formation of plaques,<sup>178</sup> whereas Cu(II) and Zn(II) are deposited in them adventitiously,<sup>761</sup> although these proposals clearly need more investigation.

In terms of treatment, chelates that remove Al from hyperphosphorylated tau<sup>762</sup> have been found to slow AD progression by ~50%,<sup>763</sup> with benefits up to 5 years,<sup>764</sup> although other reports showed negligible benefits of chelation therapy.<sup>765</sup> In summary, while pathogenic mechanisms related to iron(III)-mimetic toxicity in AD are plausible, they remain unproven,<sup>45,741</sup> and the many studies correlating Al to AD need to be investigated further to evaluate whether the relationship is causal.

## 10.2. Cadmium

There is no causal evidence of a role of Cd in AD, but elevated Cd levels have been observed in the liver of AD patients,<sup>594</sup> whereas brain levels seem unaffected,<sup>168</sup> and Cd levels have been reported to be elevated in the cerebrospinal fluid of AD patients.<sup>766</sup> Also, because Cd(II) is an important exogenous Zn(II)-mimetic metal, it deserves mentioning in this review.

Cadmium is a highly toxic heavy metal with a physiological half-life of 15–20 years, a key factor for its toxicity.<sup>767</sup> The European Union's suggested maximum allowed concentrations of Cd(II) are 0.66  $\mu\text{g}$  per gram of creatinine in the urine.<sup>768</sup> Cd intake from food (seafood, rice, and vegetables) on average leads to ~1  $\mu\text{g}$  of Cd intake per average person per day, whereas the tolerable weekly intake is approximately 2.5  $\mu\text{g}$  per kg of body mass,<sup>767</sup> but smoking or other environmental exposures from pollution, etc., may lead to toxic doses.<sup>767</sup> The most common Cd exposure is from tobacco smoke,<sup>769</sup> and observed higher levels of heavy metals in smokers,<sup>770</sup> notably 5

times higher cadmium levels in blood compared with nonsmokers,<sup>771</sup> could provide one possible reason for the correlation between AD and Cd/smoking.<sup>772</sup> Incidentally, smokers also experience hyperhomocysteinemia, a risk factor of AD.<sup>773</sup>

Cd(II) is toxic via a number of mechanisms, notably via oxidative stress<sup>774</sup> and zinc-mimetic chemistry.<sup>775,776</sup> Cadmium is below zinc in the periodic table and easily replaces it in many chemical reactions.<sup>724,777</sup> Cd(II) is larger, more polarizable, and hence a softer Lewis acid than Zn(II); it is more thiophilic (displays larger affinity for sulfur ligands) than Zn(II) and can thus displace Zn(II) in proteins where cysteine or methionine are present. Most Cd(II) is bound to cysteines in MT as part of normal detoxification.<sup>778,779</sup> Thus, Cd(II) is, together with Hg(II) also iso-electronic with Zn(II), a key contender in disruption of zinc homeostasis. As for Zn(II), bioavailable Cd(II) is always found in the divalent oxidation state, with a complete d-electron shell and a symmetric, usually tetrahedral coordination chemistry.

Related to AD, Cd(II) may interrupt tau processing,<sup>780</sup> and Cd(II) bound to MT<sup>781,782</sup> will disturb metal binding sites and could possibly increase the free Zn<sup>2+</sup> pool while impairing Zn(II) transfer to zinc proteins,<sup>532</sup> which could cause MT dysfunction contributing to zinc dyshomeostasis,<sup>783</sup> although this is currently unexplored. Alternatively, due to its zinc-mimetic properties, Cd(II) may bind directly to regulatory zinc sites affecting APP processing, causing amyloid imbalance by direct zinc substitution of regulatory Zn<sup>2+</sup> instead of affecting the size of free Zn<sup>2+</sup> pool as above. There is evidence that Cd(II) binds close to the  $\alpha$ -secretase site in APP, thus favoring amyloid production.<sup>784</sup> This site is likely to be the regulatory Zn<sup>2+</sup> site in APP, which is also located in this region.<sup>288</sup>

Importantly, dyshomeostasis of iron may contribute to increased uptake of heavy metals such as Cd(II),<sup>785</sup> and as mentioned previously, homeostasis of Cu, Zn, and Fe is intimately related, notably via APP, MT, and ceruloplasmin. Thus, risk factors can act in concert to produce toxic effects that cannot be evaluated as isolated entities. This insight is essential for understanding metal-induced neurotoxicity.

## 10.3. Lead

Lead (mainly in the Pb(II) oxidation state) is also neurotoxic.<sup>725,786</sup> It impairs neurotransmission<sup>787</sup> and has been found to impair social and cognitive skills in children<sup>788–790</sup> and long-term memory.<sup>791</sup> The main target of Pb toxicity is the brain,<sup>792</sup> in particular zinc and calcium sites in proteins,<sup>726</sup> and cognitive deficits occur after exposure at commonly encountered low doses, <75  $\mu\text{g}/\text{L}$  blood.<sup>793</sup>

Links between such early exposure and late onset of AD have been observed in primates,<sup>794,795</sup> and it is plausible that exposure during fragile CNS development could enhance risk of AD later in life,<sup>42,796,797</sup> although several studies have not found a correlation between Pb exposure and development of AD.<sup>42</sup> In young rats, the morphological and synaptic impairments by Pb(II) that could be causative have been described.<sup>798</sup> The literature of Pb exposure relating to AD was recently reviewed.<sup>42</sup>

Possible molecular pathogenic mechanisms of Pb relating to AD may include binding to glutathione and thereby induction of oxidative stress.<sup>479</sup> As for cadmium, lead is highly thiophilic and thus interferes with zinc homeostasis by disrupting Zn(II)-binding sites with soft ligands such as cysteine.<sup>799</sup> *In vitro* Pb exposure up to 50  $\mu\text{M}$  was found to correlate with



overexpression of APP and reduced activity of neprilysin, one of the zinc proteases involved in  $A\beta$  degradation.<sup>800</sup> In primates, APP overexpression from Pb exposure was recently found to be due to DNA methylation toxicity.<sup>801</sup> In other *in vivo* studies, Pb seemed to inhibit  $A\beta$  transport by the low-density lipoprotein-receptor-related protein 1<sup>802,803</sup> (LRP1; aka apolipoprotein E receptor (APOER)), which is the membrane receptor that transports apoE4-cholesterol to the neurons.<sup>804</sup> Recently, Pb(II) was also found to catalyze tau protein aggregation via binding to His-330 and His-362.<sup>805</sup>

Interestingly, MT has been found also to bind to receptors such as LRP1, probably to transfer Zn(II) and facilitate signal transduction for neuronal growth.<sup>806</sup> Adverse effects of Pb exposure are aggravated by the presence of the APOE4 allele, suggesting a correlation between Pb exposure and this protein.<sup>807,808</sup> Thus, although many mechanisms could explain a causal effect of early Pb exposure and later AD development, more studies are needed to provide such causation, which is currently not established.<sup>42</sup>

#### 10.4. Mercury

Mercury (Hg) is a highly neurotoxic element,<sup>725,809–811</sup> and accumulated evidence now identifies it as a risk factor for AD.<sup>43,812,813</sup> Major sources of Hg are fish consumption, the Hg-containing alloy amalgam in dental fillings,<sup>814</sup> some vaccines, and urban air pollution.<sup>813,815</sup> For example, amalgam may leak 1–12.5  $\mu\text{g}$  of Hg per day, with long time accumulation due to substantial retention.<sup>810</sup> It has been estimated that ~300 000 newborns may have been exposed to Hg concentrations that could cause neurological damage.<sup>816</sup>

It is known that during mouse pregnancy, Hg(0) vapor penetrates the placental barrier and damages fetal organs including the brain; MT protects against this penetration.<sup>817</sup> Hg is very BBB-penetrable (80%) and localizes in the spinal cord, of relevance to ALS,<sup>818</sup> and in the cortical brain, of relevance to AD.<sup>959</sup> A particularly toxic form, methyl mercury [ $\text{CH}_3\text{Hg}$ ]<sup>+</sup>, causes lethal neuronal damage.<sup>819</sup> [ $\text{CH}_3\text{Hg}$ ]<sup>+</sup> from seafood accumulates in astrocytes that express MT-1/MT-2,<sup>820</sup> central homeostatic cells in AD pathogenesis,<sup>429</sup> to be discussed later.

The chemical mechanisms of neurotoxicity are several: Hg(II) binds to soft Lewis base amino acids cysteine and methionine in various proteins,<sup>821</sup> notably tubulin, the structural protein component of microtubules in the cytoskeleton that are produced by tau protein, thereby inhibiting binding of guanosine triphosphate (GTP)<sup>822,823</sup> and ADP ribolyzation of tubulin,<sup>824</sup> both required for normal microtubule formation and hence cytoskeleton integrity,<sup>825</sup> thus leading to cell degeneration.<sup>826</sup> Such a cascade would also cause tau hyperphosphorylation and neurofibrillar tangles as a consequence of heavy metal exposure to the CNS,<sup>813</sup> which is indeed observed upon Hg exposure,<sup>826–828</sup> in concurrence with increased  $A\beta$  secretion,<sup>829</sup> Hg-induced  $A\beta$  aggregation,<sup>830</sup> tubulin dysfunction, and apoptosis.<sup>831</sup>

In rats, Hg has been shown to interact biologically (although probably not directly) with melatonin. Melatonin levels are depleted upon Hg exposure, while melatonin production increases.<sup>832–834</sup> The chemical interaction could occur via binding to enzymes involved in melatonin pathways or substitution of zinc in proteins leading to zinc binding to melatonin. On the other hand, Hg binds directly to selenium,<sup>520</sup> preventing selenium availability to glutathione peroxidase, thus indirectly causing further oxidative stress. Hg

may inhibit  $A\beta$  transport by LRP-1 as Pb does,<sup>802,803</sup> which could explain the effect of Hg on amyloid secretion.

Hg levels in AD brains have been estimated at 0.02–0.18  $\mu\text{g}/\text{g}$  tissue,<sup>835,836</sup> or  $[\text{Hg}] \approx 0.1\text{--}0.9 \mu\text{M}$ .<sup>813</sup> Some studies have not found any correlation between Hg from amalgam and AD,<sup>837</sup> although these concentrations of Hg are much higher than Hg concentrations (0.1 nM) found to cause axon degeneration and neurofibrillar tangles in other studies.<sup>826,813</sup> However the lack of correlation between amalgam and Hg concentrations in that study<sup>837</sup> is inconsistent with the consensus of Hg migration from amalgam from a wide range of studies,<sup>838–845</sup> suggesting erroneous monitoring of Hg.<sup>813</sup> In fact, even Hg from amalgam in mothers correlate with Hg in fetal and infant tissue,<sup>846</sup> consistent with observed Hg transfer across rat placenta.<sup>817</sup> Depending on the lipophilicity of various toxic forms of heavy metals, monitoring hair, nails, blood, and other tissue will necessarily give different results.<sup>847</sup> In addition, contents in different tissues reflect different timings and periods of exposure, namely, the pharmacokinetics.<sup>813</sup> Thus, blood concentrations, reflecting long-time exposure, of Hg, Cd, and Al were significantly elevated in AD patients, whereas Cu was elevated in the cerebrospinal fluid.<sup>766,848</sup> In contrast, patients with AD have been found to have less Hg in nails and hair compared with controls.<sup>849</sup>

Many studies have found exogenous metal exposure to enhance risk of other neurological disorders, notably in this context Parkinson's disease<sup>196,850</sup> and ALS,<sup>851–855</sup> which as discussed shares many features with AD. Increased heavy metal load has also been found in ALS patients.<sup>856–859</sup> While correlation is no longer disputed, causation is substantially more difficult to prove or disprove. However, natural metal homeostasis (Cu, Zn, Fe) provides an emerging framework for discussing exogenous metal exposure as one of many risk factors that can perturb this homeostasis and lead to neurological disorders such as AD.

## 11. NATURAL METAL CHELATORS AGAINST ALZHEIMER'S DISEASE

Given the central role of bioinorganic chemistry in AD pathogenesis, it is not surprising that many known beneficial dietary prevention strategies against AD in fact involve BBB-penetrable antioxidant and metal-chelating substances.<sup>56</sup> Many such compounds are found as natural products or well-known drugs for other purposes and can be obtained as part of a diet.<sup>860–863</sup> In addition to being antioxidant and metal-chelating with the optimal  $K_d$  and selectivity between targeted metal ions, these compounds should also be hydrophobic and capable of crossing the BBB and must be of reasonable small molecular weight and fulfill various other, typical criteria of drug-likeness.<sup>445,864,865</sup> Here, six such candidates will be discussed: lipoic acid, *N*-acetyl-L-cysteine (NAC), epigallocatechin gallate (EGCG), curcumin, melatonin, and galantamine.<sup>863,866</sup>

Lipoic acid is a coenzyme for pyruvate dehydrogenase in the mitochondria and thus plays a role in neuronal energy production. Already in the 1990s, it was reported to improve cognition of rodents,<sup>867,868</sup> and it has been in focus as a disease-modifying treatment of AD<sup>869–871</sup> and as part of dietary supplement to reduce risk of AD.<sup>863</sup> Lipoic acid contains a carboxylate functional group and two vicinal sulfur atoms, that is, both hard and soft Lewis-base donors, which should be useful in targeting both more thiophilic (Cu, Zn, Cd) and oxophilic (Fe, Al) metal ions. In its reduced form, dihydrolipoic acid, as usually found in cells,<sup>872</sup> it can chelate metal ions. It has

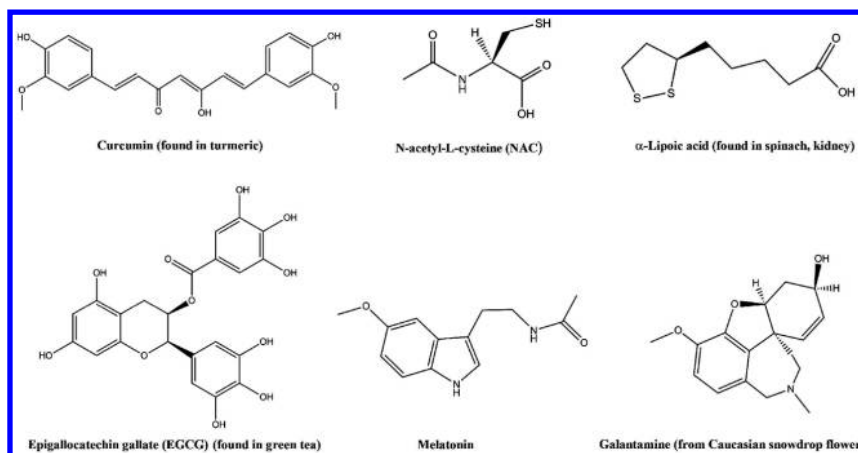


Figure 14. (Pseudo)natural antioxidants with metal-chelator function under investigation for treatment of AD.

been found to reduce oxidative stress mediated pathogenesis in AD cells.<sup>467,873</sup> Notably, together with acetyl-L-carnitine, another natural antioxidant chelator, lipoic acid may enhance  $\alpha$ -secretase activity and thus partly restore amyloid balance<sup>874</sup> and improve cognitive function of ApoE4 mutant mice.<sup>875</sup>

NAC, N-acetylated cysteine, is a simple, nontoxic compound used against cystic fibrosis and coughing, capable of breaking disulfide bonds and dissolving mucus.<sup>876,877</sup> NAC can also act as a chelator<sup>878</sup> via some of its potential donor lone pairs on thiol, carboxylate, amine, and carbonyl. NAC is a membrane-penetrable precursor to cysteine and is usually hydrolyzed to cysteine or forms disulfides with life times up to 6 h.<sup>879</sup> NAC has been shown to be beneficial in schizophrenia, possibly by inhibiting GABA-R,<sup>880</sup> and in various other neurological disorders.<sup>879</sup> NAC has been shown in trials to be beneficial to patients with probable AD<sup>881</sup> and has also been suggested as a part of dietary prevention of AD.<sup>863</sup> NAC can reduce oxidative damage<sup>882</sup> and displays anti-amyloid effects<sup>883</sup> in mouse models of AD.

EGCG is a main phenol constituent (catechin) of green tea and contains several sites for bidentate coordination of metal ions,<sup>467</sup> both via vicinal hydroxy groups and via hydroxy groups on separate rings (see Figure 14). EGCG is both BBB-penetrable and an effective M(II) chelator,<sup>884</sup> and catechins are found to bind Fe(II),<sup>885</sup> Zn(II),<sup>886</sup> and Cu(II) with associated antioxidant reactivity.<sup>887</sup> Also Fe(III)–gallocatechin complexes with variable stoichiometries have been described,<sup>888,889</sup> as well as Al(III) complexes with  $K_d \approx 10^{-8}$ – $10^{-5}$  M.<sup>890</sup> For 1:1 complexes of Zn(II) and Cu(II) catechins, formation constants of  $10^5$ – $10^6$  M<sup>-1</sup> (corresponding to  $K_d$ 's of  $10^{-6}$ – $10^{-5}$  M) were observed,<sup>891</sup> enough to sequester the free chelatable M(II) pools but too weak for outcompeting amyloids ( $K_d \approx 10^{-10}$ – $10^{-7}$  M).

As a potential treatment against AD, EGCG has been found to inhibit  $A\beta$  production in Swedish double mutant transgenic mice (mice encoded with the mutant known to cause the severe example of AD in a Swedish family), accordingly by promoting the non-amyloigenic  $\alpha$ -secretase pathway<sup>892</sup> while at the same time modulating tau pathology and cognitive decline.<sup>893</sup> Other researchers have confirmed these results and identified possible pathways of inhibition,<sup>894</sup> while some suggest that APP expression is reduced via iron chelation.<sup>467</sup>

Curcumin is found in the Indian spices turmeric and curry and is known to be an antioxidant metal chelator<sup>895</sup> that binds to a large range of biological targets, as recently reviewed.<sup>896</sup>

Epidemiological studies have found that the incidence of AD among people in their 70s was about 4–5 times smaller in India than in the US and that curry consumption correlated with this tendency.<sup>897,898</sup> While researching the beneficial effects of curry, curcumin was found to protect against  $A\beta$ -induced cognitive deficits<sup>899,900</sup> by direct binding to amyloid *in vitro* and *in vivo*.<sup>901</sup> The  $K_d$ 's for Cu(II)–curcumin have been reported to be  $\sim 10^{-6}$  M if two curcumin molecules bind each Cu(II) and  $\sim 10^{-5}$  M in 1:1 complexes.<sup>902</sup> Thus, as for EGCG, competitive 1:1 MPAC function is unlikely also for curcumin. However, despite the large Cu(II)  $K_d$  curcumin may bind *directly* to the amyloid, thereby modifying its conformation and preventing oligomerization,<sup>901</sup> although the molecular details of such inhibition need to be further explored.

Absent direct competitive binding or modifying interaction with amyloids, the benefits may also arise from targeting the expanded free pools of Cu<sup>2+</sup> (subject to Fenton toxicity) and Zn<sup>2+</sup> (possibly to reduce regulatory zinc levels) or very weakly bound metal ions, for example, in APP ( $K_d$  for zinc site  $\sim 10^{-6}$  M<sup>288</sup>). By targeting these free pools, curcumin may function upstream from amyloid production. Curcumin has been reported to suppress presenilin expression and inhibit  $A\beta_{40/42}$  formation,<sup>903</sup> apparently with IC<sub>50</sub> values smaller than  $\sim 5$   $\mu$ g/mL.<sup>904</sup> Crystal structures of curcumin binding to segments of tau protein have also been recently reported.<sup>108</sup> Other researchers have found that curcumin enhances macrophage consumption and clearance of amyloids,<sup>905–907</sup> and curcumin will reduce any heavy-metal toxicity in the neurons.<sup>908</sup> These beneficial effects of curcumin may partly explain why inhabitants of rural Northern India have some of the world's lowest age-corrected AD incidences.<sup>866</sup>

New curcumin-derivatives are among the many metal chelators currently being optimized for AD treatment,<sup>445,909</sup> and at least 10 patents have been filed in the last couple of years regarding curcumin or its derivatives in AD treatment (See Table 1).

Melatonin is a BBB-penetrable hormone involved in maintaining the circadian rhythm and hippocampus memory formation<sup>910</sup> (see Figure 14). While the day concentration is 10 times higher than at night in young individuals, the difference can almost disappear in older people due to reduced melatonin production.<sup>911</sup> Melatonin's antioxidant,<sup>912,913</sup> anti-inflammatory,<sup>914,915</sup> and antiapoptotic effects on neurological disorders are well-documented,<sup>916–919</sup> while therapeutic potential is also large due to low toxicity.<sup>920</sup>

Table 1. Patents Addressing Disease-Modifying Treatments of AD Based on the Metal Ion Hypothesis

| active substance(s)  | hypothesis   | strategy  | patent number                | filing date |
|--|--|---|------------------------------|-------------|
| Chelators  |  |   |                              |             |
| clioquinol   | clioquinol is effective against AD   | antiamyloidosis by clioquinol, B <sub>12</sub> (+carrier)                   | US 6,001,852                 | 2/1998      |
| various metal chelators  | metal chelation can resolve amyloids, enhance clearance                                    | chelators against AD  | US 6,323,218 B1              | 3/1998      |
| bathocuproine/indomethacin   | metal chelation can resolve amyloids, enhance clearance                                    | chelators against AD  | US 7,045,531 B1              | 3/1998      |
| lipophilic diesters of metal chelators                             | antioxidant metal chelators against neurological disorders                                 | chelators against neurological disorders, including AD                      | US 6,458,837 B1              | 9/1998      |
| 1,10-phenanthroline complexes                                      | metal chelation can resolve amyloids, enhance clearance                                    | chelators against AD  | US 7,704,987 B1; WO 01/07442 | 7/2000      |
| deferoxamine (DFO)   | metal chelators against neurological disorders   | DFO against neurodegeneration caused by ischemia                            | US 7,618,615 B2              | 8/2005      |
| phenolic compounds   | antioxidant metal chelators against neurological disorders                                 | antioxidant chelators against neurological disorders, including AD          | US 2003/0236202              | 4/2003      |
| N-alkyl N-phenylhydroxylamine chelators                            | metal chelation can resolve amyloids, enhance clearance                                    | chelators against AD, PD, stroke  | US 2003/0225087              | 6/2003      |
| 8-hydroxy quinoline derivatives                                    | metal chelation can resolve amyloids, enhance clearance                                    | chelators against neurological disorders, including AD                      | US 2006/0089380              | 7/2003      |
| clioquinol derivatives   | metal chelation can resolve amyloids, enhance clearance                                    | chelators against AD  | US 2006/0167000              | 10/2003     |
| metal chelators  | Zn chelation can modulate APP  | modulate interaction between metals and APP                                 | US 2004/0265847              | 11/2003     |
| amyloid-binding metal chelators                                    | metal chelators can resolve amyloids   | dual function, amyloid binding–metal chelating                              | US 2004/0204344              | 6/2004      |
| N-containing polycyclic chelators                                  | metal chelation can resolve amyloids, enhance clearance                                    | chelators against neurological disorders, including AD                      | US 2007/0185072              | 3/2004      |
| clioquinol   | clioquinol is effective against AD   | antiamyloidosis, possibly formulated with B <sub>12</sub>                   | US 2006/0074104              | 4/2005      |
| polyquinoline derivatives  | metal chelation can resolve amyloids, enhance clearance                                    | chelators against neurological disorders, including AD                      | US 2009/0227626              | 8/2006      |
| deferoxamine (DFO)   | metal chelators against neurological disorders   | nasal administration of DFO against neurological disorders                  | US 7,776,312 B2              | 10/2006     |
| 8-hydroxyquinoline derivatives                                     | metal chelation can resolve amyloids, enhance clearance                                    | antiamyloidosis for neurological disorders including AD                     | US 2008/0161353              | 9/2007      |
| various metal chelators  | metal chelation can resolve amyloids, enhance clearance                                    | chelators against neurological disorders, including AD                      | US 2009/0233893              | 5/2007      |
| multifunctional ring-containing chelators                          | antioxidant metal chelators against neurological disorders                                 | orally administered against neurological disorders, including AD            | US 2009/0105269              | 9/2008      |
| chelating agents   | antioxidant metal chelators against neurological disorders                                 | chelators against neurological disorders, including AD                      | US 2010/0234338              | 3/2009      |
| deferoxamine (DFO)   | metal chelators against neurological disorders   | nasal administration of DFO against neurological disorders                  | US 2010/0267834              | 7/2010      |
| MT Derivatives   |  |   |                              |             |
| MT   | MT dysfunction leads to neurological disorders   | direct MT to neuron to restore free/bound zinc and copper balances          | WO 03/105910 A1              | 6/2003      |
| MT-derived peptides  | MT dysfunction leads to neurological disorders   | MT peptide fragments as MPACs or for rebalancing the free/bound zinc pools  | US 2010/0166759              | 2/2007      |
| modified, selenium-containing MTs                                  | MT dysfunction leads to neurological disorders   | modified MTs formulated to higher efficiency than MTs                       | US 2009/0318333              | 3/2007      |
| antioxidant X-Cys-X-Cys-X peptides                                 | antioxidant metal chelators against neurological disorders                                 | chelators against neurological disorders, including AD                      | US 2011/0190195              | 4/2011      |
| Dietary Cocktails  |  |   |                              |             |
| zinc, vitamin C/E/B6, selenium, glutathione, various amino acids   | autism combination treatment based on MT dysfunction hypothesis                            | MT promotion by dietary cocktail of vitamins and amino acids                | US 7,534,450 B2              | 5/2007      |
| turmeric (curcuminoids), EGCG, lipoic acid, B12, vitamin C/E, etc. | AD combination treatment against oxidative stress, A $\beta$ , inflammation, and glycation | cocktail of natural ingredients targeting several or all mentioned pathways | US 2009/0143433              | 12/2008     |
| Curcuminoids   |  |   |                              |             |
| turmeric extract/curcuminoids                                      | turmeric extracts reduce amyloid more than curcumin  | formulation of turmeric extract against AD                                  | US 2010/0098788              | 10/2009     |
| curcumin–cyclodextrin  | curcumin–cyclodextrin is more effective than curcumin                                      | curcumin–cyclodextrin administration to AD patient                          | US 2010/0179103              | 6/2009      |
| curcumin prodrug derivatives                                       | intranasal administration enhances curcumin effect   | curcumin is administered intranasally to AD brains                          | US 2008/0076821              | 4/2007      |
| heterocyclic compounds   | heterocyclic compounds may inhibit A $\beta$ deposition                                    | AD treatment by oral dose of drug can delay AD                              | US 2008/0103158              | 10/2007     |
| curcuminoid  | bioavailable curcuminoids may be efficient against AD                                      | curcuminoid, antioxidant, and carrier formulation                           | US 2009/0324703              | 3/2007      |



Table 1. continued

| active substance(s)            | hypothesis  | strategy  | patent number   | filing date |
|--------------------------------|---|---|-----------------|-------------|
| Curcuminoids                   |   |   |                 |             |
| curcumin derivatives           | curcumin reduces $A\beta$ burden and oxidative stress in AD | bioavailable curcumin derivatives               | US 2006/0060644 | 6/2006      |
| curcumin derivatives           | curcumin reduces $A\beta$ burden and oxidative stress in AD | curcumin derivatives inhibit plaque formation   | US 2010/0087527 | 9/2009      |
| curcumin nanoparticles         | formulation of curcumin that enhances bioavailability       | curcumin nanoparticles against AD, cancer, etc. | US 2011/0190399 | 7/2009      |
| methylene blue curcumin analog | formulation of curcumin that enhances bioavailability       | curcumin methylene blue against AD              | US 7,906,643    | 2/2010      |

In AD patients, the melatonin levels are further reduced compared with same-age healthy individuals.<sup>921,922</sup> Early sleep disorders have been associated with AD, possibly indicating a common underlying etiology,<sup>923</sup> and light therapy may work<sup>924</sup> against AD-associated sleep disorder.<sup>925</sup> Melatonin may counter cognitive impairment,<sup>926,927</sup> and on the molecular level, it reduces amyloid toxicity<sup>916,919,928</sup> and tau hyperphosphorylation<sup>929–931</sup> and may thus be efficient against AD although the molecular mechanisms are unknown.<sup>932–934</sup>

Melatonin contains an amide functional group connected to two rotatable C–C bonds, providing potential flexibility to chelate Cu(II) and Zn(II), and chelation has been observed experimentally.<sup>935</sup> Melatonin may work as a metal chelator when inhibiting formation of soluble Cu(II)– and Zn(II)–amyloid oligomers.<sup>936,937</sup> Melatonin's antioxidant activity against  $A\beta$ -induced ROS may protect cultured neurons<sup>938</sup> and protect mitochondria of Alzheimer models of mice.<sup>939</sup> Recent work suggests that melatonin may induce heme oxygenase known to be down-regulated in AD and thus help restore iron/heme homeostasis, see section 4.8.<sup>940</sup> Melatonin also functions as an antioxidant in ALS,<sup>941</sup> probably scavenging superoxide ( $O_2^-$ ) in absence of functional SOD, in the same way it scavenges superoxide generated by amyloids.<sup>912</sup> Melatonin also protects neurons from Hg toxicity,<sup>829</sup> but whether it is by scavenging of ROS or by metal chelation is unknown. Of special interest in future developments are melatonin derivatives with improved antioxidant capabilities such as tacrine–melatonin hybrids.<sup>942,943</sup>

Galantamine, for example found in the Caucasian snowdrop (*Galanthus caucasicus*), has been used against neurological symptoms in the Soviet Union at least since the 1950s.<sup>944</sup> The compound is a potent acetylcholinesterase inhibitor,<sup>945,946</sup> approved by the FDA for AD treatment, showing beneficial effects on learning and memory<sup>947,948</sup> with a safety profile resembling that of synthetic acetylcholinesterase inhibitors currently on the market.<sup>949</sup> Importantly, galantamine has been found to be more potent and suggested to work via more mechanisms than synthetic inhibitors.<sup>950</sup> Stimulation of  $A\beta$  phagocytosis was recently suggested as a second mechanism for galantamine, which could possibly explain these observations.<sup>951</sup> Thus, this compound would be an example of a dual-function treatment targeting both acetylcholinesterase and  $A\beta$  clearance and is sold under the trade name Razadyne.<sup>65</sup>

A plausible mechanism of action of natural antioxidant BBB-penetrable metal chelators in enhancing  $\alpha$ -secretase activity may be chelation of free inhibitory divalent metal ions, reducing the pool of  $Cu^{2+}/Zn^{2+}$  bound in the APP  $\alpha$ -splicing region,<sup>67,288</sup> since the  $K_d$ 's ( $\sim 10^{-6}$  M) are too small for MPAC function ( $K_d < 10^{-8}$  M). A second possibility is that they prevent a ROS-producing toxic mode of free metal ions or

amyloids, thus reducing oxidative stress, which could also improve amyloid balance, as explained in section 5.

## 12. COMBINING THE HYPOTHESES: A BIOINORGANIC VIEW OF ALZHEIMER'S DISEASE

### 12.1. Dysfunction of Proteins Involved in Metal Homeostasis: MT as an Example

AD is a complex disorder with genetic, environmental, and lifestyle-related risk factors, with distinct pathological features, and with age as a substantial trigger. Because of this, any pathogenic theory of AD needs to not only explain the many risk factors and beneficial factors but also explain how the aging brain becomes a target of AD as a function of these risk factors. Furthermore, although  $A\beta$  oligomers are neurotoxic and their hydrophobicity correlates with oligomerization and membrane interaction abilities as well as toxicity,<sup>239</sup> it is not currently clear whether such toxicity is the cause of neurodegeneration in AD, for example, via signaling of apoptosis by  $A\beta$  binding to mitochondrial alcohol dehydrogenase,<sup>498</sup> and if so, whether it implicates metal ions only in the formation or as actual constituents of these toxic forms or whether amyloid toxicity is secondary to underlying gradual dyshomeostasis that would suggest Fenton toxicity or regulatory protein dysfunction (e.g., loss of metalloprotein function by functional metal ion deficiency due to reduced bound natural metal pools) as primary toxic modes.

The role of metal ions in defining AD etiology is rapidly emerging,<sup>11,12,25,68,141</sup> and the metal ion hypothesis can, as described, explain most pathological features and risk factors linked to AD, although much remains to be established. In particular, as has been the focus of this review, zinc homeostasis, the balance between the free  $Zn^{2+}$  and bound Zn(II) pools, affects all the pathways known to be closely associated with AD, including the two hallmark molecular features, amyloidogenesis and tau pathology. Dyshomeostasis of metal ions occurs mainly within those areas of the brain (hippocampus, cerebral cortex<sup>10,166,311</sup>) where  $A\beta$  accumulates and AD first sets in.<sup>384,952</sup> The amyloid cascade hypothesis, in contrast, does not currently in itself explain the localized  $A\beta$  accumulation, because  $A\beta$  is produced throughout the brain.<sup>8,79</sup> However, in terms of zinc homeostasis, it is natural that AD progresses from the ZEN and astrocytes in the cerebral cortex.

As discussed in this review, disturbed zinc homeostasis occurs via dysfunctional ZnTs, MTs, or ZIP transporters.<sup>10,144</sup> Genetic errors or polymorphisms can contribute to changes in zinc binding affinity of these proteins or change their production–clearance balances and may gradually change the Zn(II)/ $Zn^{2+}$  balance in ZEN and astrocytes that maintain CNS homeostasis and thus express most of the MT-3.<sup>535,953</sup> Furthermore, post-translational modifications from, for example, oxidative stress or exogenous exposure may impair the

function, stability, and clearance of these metalloproteins directly. For example, ROS exposure leads to increased free  $Zn^{2+}$  pools in normal astrocytes but not in MT-3-null astrocytes.<sup>954</sup>

The hypothesis that dysfunctional or insufficient MT is a partial cause of AD, leading to increased free  $[Zn^{2+}]$ , partly retained in vesicles,<sup>170</sup> lack of neuromodulation, and free  $Zn^{2+}$ -induced amyloid aggregation, was first put forward by Bush.<sup>68</sup> It has been accompanied by other theories of MT dysfunction as a central cause of zinc imbalances in ALS.<sup>701,712</sup> Zinc dyshomeostasis is a plausible underlying pathogenic cause of age- and AD-related biochemical changes which is simple and has broad explanatory power and can explain the failure of anti-amyloid drugs that target only one of many zinc proteases involved in  $A\beta$  imbalance.<sup>65,136,137</sup>

Age-correlated impairment of zinc homeostasis most likely contributes to senescence.<sup>955</sup> Zinc dyshomeostasis and MT suppression are correlated with telomere shortening,<sup>643</sup> and free  $Zn^{2+}$  is an underlying regulator or active site ingredient of most of the proteins discussed in this review, including the zinc proteases that control the amyloid production–clearance balance. Expression of inflammatory interleukins (IL) and MT-1/MT-2 increases with age and AD.<sup>956</sup>

Astrocytes maintain brain homeostasis by expressing MTs, SOD-1 and SOD-3, and amyloid-degrading metalloproteases,<sup>94</sup> and have been implicated as dysfunctional in neurological disorders.<sup>428,429</sup> MT-3 plays little role in oxidative-stress/inflammation but an important role in zinc and copper homeostasis and cell regeneration, whereas MT-1/MT-2 are antioxidants in particular in the astrocytes<sup>535</sup> (this is probably why MT-1/MT-2 deficient mice are less resistant to ALS induced by G93A mutations in Cu,Zn-SOD<sup>957,958</sup>). MT-3 dysfunction will necessarily disturb zinc and copper homeostasis, impairing astrocyte and ZEN function.<sup>10,144</sup>

Nasally administrated Hg,<sup>959,960</sup> Mn,<sup>961</sup> or other metals<sup>962</sup> may accumulate in the brain and induce and bind to MT,<sup>316,963,964</sup> and thiophilic metals such as Hg(II) easily substitute Zn(II) in MT.<sup>965,966</sup> This enhances the free  $Zn^{2+}$  pool and reduces the bound Zn(II) pool, while the remaining Zn–S bonds are perturbed, most likely to the effect of altering the  $K_d$ 's that are essential for MT's buffering function.<sup>967</sup> Various stress inducers such as ROS and exogenous metals perturb zinc homeostasis via MT.<sup>260</sup> The abnormal MT expression in AD<sup>580,592,535</sup> may in part be related to such insults to the CNS,<sup>441,968,969</sup> although stress-induced interleukins,<sup>539,551</sup> glucocorticoids,<sup>541,552,553</sup> and oxidative stress<sup>555</sup> possibly from other pathogenic events also induce MTs separately. If MTs become dysfunctional, it is plausible that astrocyte homeostatic capabilities are substantially reduced.

One seemingly paradoxical observation is Cu- and Zn-profile-based monitoring of MT-1/MT-2 showing reduced protein levels in AD, where other researchers have observed elevated levels with nonmetal-probing methods,<sup>592,593</sup> and the study also revealed that MTs in AD brains are more oxidized than in healthy brains.<sup>970</sup> This is however consistent with MTs being monitored that have lost their metal ions, that is, oxidized thionein apoproteins that may be dysfunctional,<sup>535</sup> consistent with the view presented here that metal ion dyshomeostasis is essentially a shift from bound M(II) to free  $M^{2+}$  pools. Also, expression of MT-3 is periodical upon neuronal insults and may decrease in proportion to degeneration of ZEN or astrocytes,<sup>591</sup> whereas the resulting free  $Zn^{2+}$  and inflammation will induce MT-1 and MT-2 in remaining neurons.<sup>971</sup> Free  $Zn^{2+}$  in

extracellular space may potentially lead to amyloid formation,<sup>176,179,183,184</sup> and arrest of  $Zn^{2+}$  in synaptic vesicles may impair neuromodulation by  $Zn^{2+}$ .<sup>261,262,266</sup> Furthermore, caspases, important mediators of apoptosis, the end stage of neurodegeneration, are induced if Zn(II) is not available in intracellular metalloproteins.<sup>972</sup>

MT dysfunction may cause tau hyperphosphorylation, a pathological feature of AD shared by other neurodegenerative diseases that all pathogenic models should explain: If MT cannot transfer Zn(II) to zinc-fingers, MSOT (mammalian suppressor-of-tau pathology) could be dysregulated, which could disturb tau phosphorylation balance.<sup>973</sup> Alternatively, the elevated free  $Zn^{2+}$  may directly enhance hyperphosphorylation of tau and release it from microtubules.<sup>974</sup> Recently it was shown that free  $Zn^{2+}$  causes tau phosphorylation via activation of kinase pathways (MAPK/ERK).<sup>975</sup>

A rationale for observed  $\alpha$ -secretase enhancing function of antioxidant chelators such as curcumin, EGCG, and lipoic acid is their free-metal chelating function that may clear free metal ions, thus reducing the burden of excessive free inhibitory  $Zn^{2+}$  in regulatory sites such as in the APP  $\alpha$ -secretase cleavage region. This is also more consistent with their relatively weak metal-binding properties ( $K_d \approx 10^{-6}$  M), which are not sufficient to competitively strip Cu(II) and Zn(II) from  $A\beta$ -metal complexes ( $K_d < 10^{-8}$  M) as MTs can. This would suggest a new focus on targeting regulatory metal ions in APP instead of amyloids directly as is normally sought with MPACs.

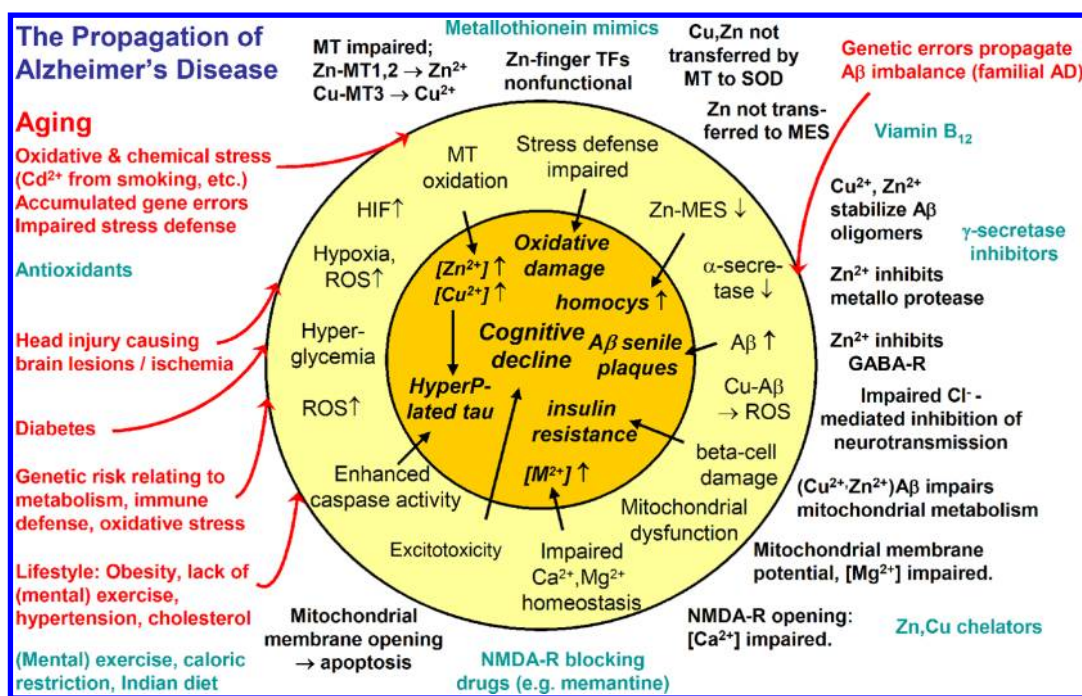
## 12.2. Converging toward Apoptosis

Apoptosis is a necessary process of CNS development, and its disturbance can cause mental disorders.<sup>729</sup> The intrinsic mitochondrial pathway of apoptosis,<sup>976</sup> if triggered by metal dyshomeostasis, seems to combine many of the pathological features observed in neurological disorders.<sup>916</sup> Apoptosis is most likely triggered when the mitochondria are insulted by further direct stress leading to shutdown of the respiratory chain and expulsion of cytochrome *c*, an iron/heme-containing protein complex that is an integral part of respiratory chain, as an early suicide event.<sup>13,487</sup> This then leads to caspase-mediated apoptosis and mitochondrial membrane permeability governed by apoE transport proteins and  $Ca^{2+}$  concentration.<sup>911</sup> Before that threshold, conditions may worsen gradually, as in MCI. The gradual buildup of stress followed by triggering events has been emphasized in relation to oxidative stress as an early cause of AD.<sup>491</sup>

ApoE, which transports cholesterol to mitochondria, is highly concentrated in astrocytes,<sup>977</sup> and impaired transport to mitochondria due to toxic exposure or mutations in ApoE might set an earlier stage for stress-induced apoptosis, thus increasing risk of triggering AD. Mitochondrial dysfunction in astrocytes due to apolipoprotein mutations may therefore be a rationale for understanding these genetic risk factors.

The fundamental role of Zn(II)/MT in preventing apoptosis<sup>539,972</sup> is evident from the research into zinc-dependent (class I, IIa, IIb, and IV) histone deacetylases that prevent apoptosis. The corresponding histone deacetylase inhibitors that are currently being developed as apoptosis-inducing cancer treatments<sup>978</sup> are efficient zinc chelators and, consistent with the findings presented in this review, show promising use as treatments of neurodegenerative diseases.<sup>979</sup>

Cytochrome *c* release and other symptoms of neuronal apoptosis in experimental stroke models can be inhibited not only by melatonin<sup>980,981</sup> but also the carbonic anhydrase



**Figure 15.** A bioinorganic view of Alzheimer's disease progression, with a focus on zinc and copper, reconciling the amyloid cascade hypothesis (as seen when starting from the upper right) with the oxidative stress (upper left) and metal ion hypotheses. Risk factors are colored in red, beneficial factors are colored in cyan-blue. A possible causal relationship to iron dyshomeostasis via the ferroxidases, ceruloplasmin, and APP is given in the text.

inhibitor methazolamide.<sup>982</sup> This compound contains significant metal chelator functions, in particular with sulfonamide groups, which have been shown to coordinate Zn(II).<sup>983,984</sup>

The most widely used treatment of AD is currently still acetylcholinesterase inhibitors such as donepezil and tacrine.<sup>860</sup> However, underlying causes of the acetylcholine neurotransmitter shortage, which leads to cognitive decline,<sup>62</sup> must be described in a unified view of AD. The most obvious explanation for acetylcholine deficiency in AD according to the MT/Zn dysfunction hypothesis is that free Zn<sup>2+</sup> inhibits choline acetyltransferase<sup>985</sup> or pyruvate dehydrogenase, which produces acetylcoenzyme A.<sup>986</sup>

### 12.3. The Zinc Cascade: An Example of Metal-Based Etiology

From the synthesis of observations discussed above, a cyclic pathogenic etiology can be constructed, as shown in Figure 15. It is based on the current emerging consensus of a self-perpetuating cascade, spiraling toward AD as the endgame of a gradual process often spanning decades, and similar views on AD have been suggested for calcium homeostasis,<sup>117</sup> zinc and calcium dyshomeostasis,<sup>354</sup> and oxidative stress.<sup>88</sup> The significance of each input is not yet well-established and may be the subject of future investigation. It is a theory based on a collection of all observations discussed in this review and as such a minimal model that will need to be corrected. The chronology is also not absolute, as evident from the multiple feedbacks discussed in this review. The cascade stresses the absence of simple, linear etiologies and emphasizes, as do other researchers,<sup>354,377,987</sup> that a systemic model is required in order to account for genetic, lifestyle, and environmental factors as they progress over time and lead to AD, possibly after crossing critical thresholds in, for example, ROS levels,<sup>491</sup> Aβ concentrations, or free metal concentrations such as Zn<sup>2+</sup>, Ca<sup>2+</sup>, or Fe<sup>2+</sup>.<sup>354,377</sup> A spiral etiology has been suggested from

studies in calcium dyshomeostasis<sup>117</sup> and a positive feedback loop between oxidative stress and the amyloid cascade also supports the cyclic model.<sup>88,508</sup> The model does not provide any weight of importance to any of the steps; these weights will hopefully be determined in the future. The AD stage can be envisioned in the center of the model, with mild cognitive impairment and preclinical phases approximately correlating with pathologies of the outer circle in Figure 15.

- (1) There is a genetic background to AD: Mutations related to Aβ balance, metabolism, immune defense, or stress-response, including metal homeostasis, may impair the robustness of the homeostatic system, notably in astrocytes, reducing resistance to post-translational modifications from later stress-inducing events.<sup>48–52</sup> Calcium homeostasis was the first established genetic risk factor relating directly to metals via presenilin,<sup>368,369</sup> although recently, presenilin was also linked to copper and zinc transport.<sup>154</sup> Genetic risk relating to APP processing may affect metal dyshomeostasis via APP's ferroxidase function and Zn<sup>2+</sup>- and Cu<sup>2+</sup>-regulatory sites.<sup>290</sup> Various genetic risk factors become active at various stages of life, some possibly during early CNS development<sup>729</sup> (presenilin may have a role in fetal neurogenesis<sup>988</sup>) and some from later brain trauma<sup>7</sup> or chemical exposure<sup>41–43,47</sup> and may lead to initiation of the spiral cascade in Figure 15.<sup>4,32</sup>
- (2) Life-style-related risk factors such as antioxidant deficiency,<sup>55</sup> obesity,<sup>35,36</sup> hypercholesterolemia,<sup>39,40</sup> passivity,<sup>57,61,56</sup> and smoking<sup>34,772</sup> may aggravate these risk factors by challenging metabolic networks, the already weakened stress response, and overall neuronal capacity. They all relate to metal ion dyshomeostasis, notably via zinc, as discussed in section 7. Long-term stress associated with an unhealthy life style may gradually



- reduce mitochondrial efficiency and stress response, as implied by the increased expression of stress-related proteins during senescence,<sup>629</sup> thus increasing risk of AD.<sup>13,71,72,486,487</sup>
- (3) Long-term or event-based insults from, for example, head trauma, smoking, or chemical exposure are known risk factors of AD<sup>7,27,38,41</sup> and may further weaken the stress response via local neuronal damage, oxidative stress, and mitochondrial inefficiency.<sup>63,70,357</sup> Insults may cause post-translational modification (oxidation, exogenous metal binding) of metal-homeostatic and stress-related proteins such as SOD, ceruloplasmin, or MT and modify amino acids binding to metal ions,<sup>568,569</sup> for example, nitrosative stress reducing bound Zn(II) used for transfer to proteins.<sup>989</sup>
  - (4) As an example, exogenous metals (Al, Hg, Pb, Cd) may be absorbed in the CNS,<sup>960–962</sup> accumulated in the spinal cord and motor neurons (ALS)<sup>818</sup> or in the olfactory bulb, hippocampus, and remaining cortical brain (AD),<sup>43,959</sup> associated with AD pathogenesis.<sup>10,73,384,952</sup> Tau phosphorylation and disruption of microtubules as well as A $\beta$  imbalances may be aggravated by exposure (see section 10).<sup>824,826,827,831</sup> Soft Lewis acids may inhibit zinc-dependent enzymes such as MES,<sup>692</sup> required for methylation pathways, and SAM, leading to hyperhomocysteinemia, apoptosis, and tau pathology,<sup>43,678</sup> and could possibly bind the known regulatory Zn<sup>2+</sup> sites in APP.<sup>290</sup> Al is instead a hard iron(III)-mimetic Lewis acid<sup>44,731</sup> and a risk factor in AD<sup>44–46,730,739,741</sup> suggested to stabilize iron-regulatory proteins and cause elevated free iron levels and Fenton-based oxidative stress and contribute to an “iron cascade” also accelerating neuronal death.<sup>144,376</sup>
  - (5) Mutations or stress-induced post-translational modifications of stress-related metalloproteins such as ceruloplasmin, MT, or SOD may impair protein function<sup>963,990</sup> or even produce toxic gain of function, as in ALS.<sup>702–704</sup> For example, Zn-MT-1/MT-2 levels in AD livers are reduced despite brain up-regulation,<sup>594</sup> suggesting that MTs exported to the liver are reduced in number or modified to bind less zinc. Many of these mutations and modifications are currently unknown and will change the risk and the onset of AD.
  - (6) Zinc dyshomeostasis is associated with AD<sup>10,11,144,148</sup> and most likely results from mutations and modifications of proteins involved in zinc homeostasis. Brain homeostasis is governed by astrocytes, zinc homeostasis is governed by MTs, particularly expressed by astrocytes, and astrocytes are central cells in AD pathogenesis.<sup>429</sup> Dysfunctional MTs would increase the free Zn<sup>2+</sup> pool and reduce the bound, transferable Zn(II) pool,<sup>532</sup> thus increasing free Zn<sup>2+</sup> between synapses in neuro-pils,<sup>146–148</sup> while global zinc levels are reduced or unchanged in the brain,<sup>152,168</sup> due to redistribution toward extracellular deposits. As a compensation, MT-1/MT-2 is up-regulated by free Zn<sup>2+</sup> and by other stress factors<sup>527,539,541,554</sup> as observed in AD.<sup>581,592,593,583</sup> MT-3 regulation is initially periodic upon neuronal insult,<sup>591</sup> as described in section 6, and may be chronically down-regulated in the advanced stage of AD if astrocytes, a possible target of early pathogenesis,<sup>429</sup> become dysfunctional.
  - (7) As an example, modifications of MT may disturb the vital balance between bound Zn(II) available for transfer to active sites and free regulatory and vesicular Zn<sup>2+</sup>.<sup>10</sup> The distinction between bound and free pools of metal ions seems essential to understand AD pathogenesis.
    - (A) The  $\alpha$ -secretases (Figure 3) are membrane zinc proteases.<sup>8,84</sup> Lack of Zn(II) in the  $\alpha$ -secretase active site could be caused by impaired transfer from MT or by mutations or post-translational modifications that reduce Zn-binding affinity. Lack of Zn(II) will impair the nonamyloidogenic  $\alpha$ -secretase pathway, leading to A $\beta$  accumulation.
    - (B) The equilibrium between metallic holo-SOD-1 and holo-SOD-3 and their demetalated apoforms is shifted toward the latter if Zn(II) is unavailable,<sup>957,958</sup> leading to gain of toxic function in some cases.<sup>273,705</sup> This may cause ALS-like pathogenesis, and oxidative stress in AD<sup>11,12</sup> could partly be due to this and partly due to the toxicity of various forms of A $\beta$ /Cu(II)<sup>80,336,338</sup> that begin to accumulate as zinc moves from MT-bound pools to free pools, since free Zn<sup>2+</sup> is a central inducer of amyloid oligomerization.
    - (C) Absence of MT-governed Zn(II) transfer to active sites of neprilysin, insulin-degrading enzyme, and matrix metalloproteinase, essential to degrade A $\beta$  extracellularly,<sup>164</sup> decisively disrupts the A $\beta$  production–clearance balance.
    - (D) Absence of Zn(II) transfer from MT to Zn(II)-dependent MES causes disruption of methylation pathways, hyperhomocysteinemia, and impairment of methylation of protein phosphatase 2A, which then increases tau phosphorylation.<sup>43,991–994</sup>
    - (E) Zn<sub>7</sub>-MT2 has been shown to substitute Cu(II) with Zn(II) and prevent toxicity and aggregation of Cu(II)–A $\beta$  10 times more efficiently than MT-3.<sup>607</sup> The absence of this function, normally likely to be mediated by the astrocyte synthesis of MT-2 (which is up-regulated in AD despite the overall neuron degeneration) could thus possibly aggravate the formation of soluble Cu(II)–A $\beta$  oligomers involved in producing the toxic forms of the amyloids.
  - (8) Free Zn<sup>2+</sup> and Cu<sup>2+</sup> (now no longer bound to MT) can provide a diagnostic tool for disease progression, for example, the Cu/MT ratio,<sup>622</sup> and these free ions may lead to A $\beta$  buildup:
    - (A) Free Zn<sup>2+</sup> and Cu<sup>2+</sup> may initiate oligomerization of A $\beta$ , stabilize oligomers, and prevent their degradation,<sup>176–182,174,189,190</sup> thus leading to A $\beta$  oligomer buildup. One plausible structure–function correlation uniting many A $\beta$ -region APP mutations that cause familial AD (Dutch (E22Q), Italian (E22K), Arctic (E22G), Iowa (D23N), English (H6R), and Tottori (D7N)) with the metal ion hypothesis is the common tendency of mutations and metal ions to neutralize and potentially increase the hydrophobicity of the negatively charged amyloids that may otherwise be resistant to oligomerization.
    - (B) APP contains both regulatory Cu(II) and Zn(II) sites, with  $K_d$  of  $\sim 10^{-8}$  M<sup>339</sup> and  $\sim 10^{-6}$  M,<sup>288</sup> respectively. Free Zn<sup>2+</sup> may bind to the  $\alpha$ -cleavage

Table 2. Genetic Risk Factors of AD and Their Possible Disease-Modifying Mechanisms and Relations to Metal Homeostasis<sup>a</sup>

| risk factor  | functions   | Genetic Risk Factors  | possible early pathogenesis  | possible advanced pathogenesis                               | possible terminal pathogenesis |
|--|---|---|--|--|--------------------------------|
| chromosome 19 ApoE4 allele   | weakened uptake of cholesterol to neuronal mitochondria and clearance of A $\beta$  | early mitochondrial dysfunction, impaired A $\beta$ -clearance via membrane transport   | oxidative stress in energy-inefficient mitochondria  | apoptosis, AD  |                                |
| chromosome 1 + 14 presenilin-2 and -1                                | part of the $\gamma$ -secretase complex that produces A $\beta$   | possibly disturbs A $\beta$ production—clearance balance  | amyloid buildup  | oxidative stress, mitochondrial dysfunction, excitotoxicity? |                                |
| chromosome 1 + 14 presenilin—metal transport                         | calcium dyshomeostasis, Cu/Zn dyshomeostasis  | defective Ca <sup>2+</sup> signaling, has been linked to Cu/Zn transport  | mitochondrial damage, caspase-activated apoptosis  | apoptosis, AD  |                                |
| chromosome 21 abnormal APP (amyloid balance)                         | possibly due to mutations near Cu or Zn regulatory sites or in zinc protease APP cleavage sites; some mutations in the A $\beta$ sequence range of APP may enhance charge neutrality and hydrophobicity | APP processing shifted toward amyloidogenesis; or more hydrophobic amyloids produced if mutated in the A $\beta$ region of APP. | amyloid buildup  | oxidative stress, mitochondrial dysfunction, excitotoxicity  |                                |
| chromosome 21 abnormal APP (ferroxidase and iron homeostasis)        | possibly due to mutations near Zn regulatory site or Fe(II) substrate site  | APP ferroxidase activity impaired   | function Fe(II/III) deficiency, Free Fe <sup>2+</sup> , Fenton chemistry                             | astrocyte dysfunction, neurodegeneration                     |                                |
| chromosome 11 picalm   | phosphatidylinositol-binding clathrin assembly protein  | impaired clathrin mediated endocytosis  | possible link to iron homeostasis  | oxidative stress, mitochondrial dysfunction                  |                                |
| chromosome 8 clustrin (CLU)  | weakened clearance of A $\beta$ involved in inflammation  | impaired A $\beta$ clearance via membrane transport   | oxidative stress in energy-inefficient mitochondria  | apoptosis, AD  |                                |
| chromosome 2 BIN1  | bridging integrator 1 (BIN1)  | impaired clathrin mediated endocytosis  | impaired membrane transport, neuronal homeostasis  |  |                                |
| genes involved in zinc dyshomeostasis: (presenilin:)                 | lack of Zn <sup>2+</sup> in synaptic vesicles of ZEN, impaired metabolism and neuro-modulation?   | oxidative stress from Cu,Zn-SOD without Zn(II); excitotoxicity in ZEN, astrocytes?  | disruption of Zn <sup>2+</sup> -induced protease activity and inhibition of mitochondrial aconitase? | amyloid cascades from ADAM dysfunction?                      |                                |
| genes involved in neuroinflammation and immune response: (CRI, CLU?) | weakened response to neuronal insults?  | brain inflammation is tightly linked to zinc and MT function  | impaired inflammatory pathways may aggravate dyshomeostasis?   |  |                                |

<sup>a</sup>Explanations and references are given in section 12.3.

Table 3. Environmental and Life-Style Risk Factors and Beneficial Factors of AD, Their Possible Mechanisms, and Relations to Metal Homeostasis<sup>a</sup>

| risk factor                 | functions  | possible early pathogenesis  | possible advanced pathogenesis   | possible terminal pathogenesis   |
|-----------------------------|--|--|--|--|
| oxidative stress            | damage to DNA and proteins   | metalloprotein dysfunction (e.g., oxidized cysteines), and <i>Aβ</i> imbalance           | mitochondrial dysfunction  | apoptosis, AD  |
| aging                       | reduced stress defenses, changes in life style, metal homeostasis, etc.                      | oxidative stress, accumulation of gene errors and chemical insults                       | see oxidative stress   |  |
| high-caloric intake         | higher metabolism causes premature aging and stress  | see aging  |  |  |
| obesity                     | accelerated metabolism, aging; higher retention of exogenous metals                          | see oxidative stress   |  |  |
| ischemia, brain lesions     | hypoxic lesions leading to oxidative stress  | disturbed homeostasis; oxidative stress from hypoxic lesions in neurons                  | zinc dyshomeostasis, see oxidative stress  |  |
| diabetes                    | impaired energy metabolism   | impaired cholesterol transport to mitochondria; dysfunctional MT; disrupted cytoskeleton | oxidative stress in energy-inefficient mitochondria oxidative stress; lack of Zn-transfer? | ALS if exposure to spinal cord/motor neurons, AD if exposure to cerebral cortex? |
| exogenous exposure          | bind ApoE4; disrupt zinc or iron homeostasis; bind neurotubulin; inhibit GTP-tubulin binding |  |  |  |
| urban air pollution         | heavy metal toxification   | see exogenous exposure   |  |  |
| smoking                     | Cd exposure  | see exogenous exposure   |  |  |
| hypertension                | increased risk of hypoxic lesions  | see ischemia   |  |  |
| hyperhomocysteinemia        | impaired methylation pathways (MES/SAM)  | apoptosis; reduced macrophage <i>Aβ</i> consumption; elevated $Ca^{2+}$ levels           | excitotoxicity; impaired <i>Aβ</i> clearance and apoptosis?                                |  |
| $B_{12}$                    | enhanced methylation pathways (MES/SAM)  | see hyperhomo-cysteinemia  |  |  |
| selenium                    | detoxifies heavy metals; ingredient in antioxidant glutathione peroxidase                    | see exogenous exposure, see oxidative stress   |  |  |
| vitamin C/E                 | antioxidants   | see oxidative stress   |  |  |
| fish                        | contain vitamin A, C, and selenium   | see oxidative stress, see selenium   |  |  |
| antioxidant metal chelators | for example, curcumin (turmeric, curry), EGCG, lipoic acid                                   | see oxidative stress   |  |  |
| exercise                    | lower resting metabolism   | see aging, see oxidative stress  |  |  |
| mental activity             | efficient mitochondrial energy production and more ZEN in cerebral cortex/hippocampus        | less oxidative stress, less sensitive to impaired ZEN                                    | reconstitute $\alpha$ -secretase activity by stripping excessive inhibitory $Zn^{2+}$ ?    | resolvate amyloid oligomers to enhance their degradation                         |
| caloric restriction         | lower resting metabolism   | see aging, oxidative stress  |  |  |
| Indian diet                 | curcumin   | see antioxidant metal chelators  |  |  |

<sup>a</sup>Explanations and references are given in section 12.3.



site of APP and prevent nonamyloidogenic  $\alpha$ -secretase activity, thus shifting APP cleavage toward  $A\beta$  production, leading to further  $A\beta$  buildup.<sup>67,288</sup> Free  $Zn^{2+}$  may also inhibit APP ferroxidase activity at this stage,<sup>290</sup> possibly causing functional iron deficiency resembling ceruloplasmin dysfunction,<sup>410,411,422</sup> although the chronology of the pathogenesis of various metal ions remains to be determined.

- (C) Active site Zn(II) transferred from functional MT allows extracellular metalloproteases to degrade  $A\beta$ , whereas free regulatory  $Zn^{2+}$  instead inhibits  $A\beta$  degradation by extracellular metalloproteases produced by the astrocytes, notably neprilysin, which is considered the main  $A\beta$  protease<sup>97,98</sup> targeting both monomers and oligomers.<sup>99</sup> This  $Zn^{2+}$  inhibition may impair  $A\beta$  clearance and leading to further  $A\beta$  buildup.<sup>94,292</sup>
- (9) The excess free  $Zn^{2+}$  in local areas around neuropils may lead to  $Zn^{2+}$ -induced hyperphosphorylation of tau,<sup>301</sup> and  $Zn^{2+}$  subsequently remains localized in the neurofibrillar tangles.<sup>302</sup>
- (10) Disrupted zinc homeostasis and elevated free  $Zn^{2+}$  may also lead to general accelerated aging processes and apoptosis by enhancing telomere shortening<sup>641–643</sup> or weakening apoptosis suppression,<sup>539,972</sup> for example, via zinc-dependent histone deacetylases.<sup>978,979</sup>
- (11) AD spreads from the cortical brain where ZEN are present that are central to memory formation.<sup>10</sup> These neurons depend vitally on MT for maintaining homeostasis and stress control, and dysfunctional astrocytes (which are main producers of MT and are targets of exogenous metal exposure<sup>820</sup>) could contribute to their degeneration<sup>25,174</sup> and are increasingly in focus as primary cells involved in neurological disease.<sup>429</sup> The hippocampus is a main stage of AD<sup>4,8,10</sup> and a main stage of metal ion dyshomeostasis.<sup>10,166,311,405,536</sup> MT-3 has been found to reduce neurodegeneration in mouse hippocampus.<sup>605</sup>
- (12) Retainment of  $Zn^{2+}$  in vesicles for co-release with glutamate disturbs neuromodulation by lack of NMDA/GABA receptor inhibition.<sup>10,262,264,282–284</sup> Free  $Zn^{2+}$  remains in neuropils.<sup>146–148</sup> Without  $Zn^{2+}$  being co-released, glutamate is not modulated,<sup>25</sup> causing excitotoxicity in gluzineric neurons located in the cerebral cortex, in particular the limbic system.<sup>264,285</sup> This leads to cognitive deficits, and the excess free  $Zn^{2+}$  ultimately leads to neuronal necrosis and apoptosis.<sup>10,298–300</sup> Free  $Zn^{2+}$  down-regulates mitochondrial energy production, for example, by aconitase,<sup>634,638</sup> and inhibits cytochrome *c* oxidase,<sup>635–637</sup> the terminal Cu- and Fe-containing protein in the respiratory chain, an example where Cu, Zn, Fe, metabolism, and oxidative stress converge. While free  $Zn^{2+}$  inhibits aconitase, MT possibly contributes to aconitase activity by transfer of Zn(II), as observed in mice.<sup>633</sup>
- (13) Zinc dyshomeostasis, a common feature of diabetes types I and II,<sup>647,650–653</sup> also leads to impaired structural integrity of insulin hexamer<sup>651,659,660</sup> and lower glucose utilization, causing insulin resistance.<sup>384,645</sup> These diabetes-like symptoms correlate with AD because they are both to some extent correlated by the same underlying variable, zinc dyshomeostasis.<sup>648</sup>

- (14) The elevated  $A\beta$ -oligomer concentration induced by the growing free  $Zn^{2+}$  pool adds to the neurotoxic cascade by interfering with the mitochondria and further impairing energy metabolism,<sup>122,123,125</sup> reducing the ability to retain the ion gradients. Possibly, this (as claimed by the amyloid cascade) is a critical trigger of self-perpetuating pathogenic cycle (Figure 15), that is, further ROS generation, MT dysfunction, elevated free  $Zn^{2+}$  and depressed bound Zn(II), further amyloid imbalance, further mitochondrial insult, and further ROS generation. A threshold or trigger succeeded by accelerated pathogenesis may explain the rapid disease progression of AD.

#### 12.4. Further Comments on the Pathogenesis

The zinc cascade presented above is not complete, and while the individual facts are settled, their causal relationships are currently hypothetical. Also, the zinc cascade is not complete because cascades relating to, for example, copper, iron, and oxidative stress have been down-played but must be incorporated for completeness. Together, the metal ion hypothesis, as exemplified by the zinc cascade above, may explain why one-dimensional treatments such as anti-amyloid drugs have so far been of limited use in AD therapy,<sup>65,136</sup> since they do not affect the underlying pathogenic pathways described.

During aging, one may say that the organism faces a metal-homeostatic challenge relating to increased free metal ion pools that provide oxidative stress and metabolic challenges to the mitochondria. Given zinc's essential role in controlling apoptosis, telomere shortening, mitochondrial energy production, and transcription via zinc-fingers, zinc is likely to be a central player in aging processes, and its dyshomeostasis would disturb these processes. The zinc cascade is an example of a minimal model that could explain in a systemic way genetic, lifestyle, and environmental risk factors and the pathological changes observed in AD and reconcile it with the accelerated aging concepts. As a minimal model, it requires expansion and further justification, and certain steps are likely to be less relevant than others. Some of the possible implications are collected in Tables 2 and 3, showing genetic and lifestyle/exogenous risk factors and their possible, hypothetical relations to metal ion homeostasis. Any future more refined theory of AD pathogenesis should probably attempt to account for many of these risk factors as well.

Other cascades arising, for example, from copper and iron dyshomeostasis will display pathogenic features that overlap substantially with each other and with the zinc cascade described above, for example, via Cu,Zn-MT-3, multicopper  $Fe^{2+}$ -oxidizing ceruloplasmin, Cu- and Fe-containing cytochrome *c* oxidase, and Cu,Zn-SOD-1. Dysfunctional metalloproteins caused by mutations or post-translational modifications that impair  $K_d$ 's by modifying the metal binding sites may be key targets in neurodegeneration, but whether any of the three metals, Fe, Cu, or Zn, is more fundamental in causing dyshomeostasis is unclear, but the consequence of enhanced free metal pools should be the same, as also discussed with respect to iron.<sup>377</sup> Also, the various metal ions display different pathways of amyloid oligomerization and fibrillation.<sup>755</sup> It should be a key goal in future research to resolve these metal ion cascades in time and space and to unite genetic risk factors with environmental and lifestyle risk factors in a systemic model that can explain both the familial and the sporadic cases of the

disease, with explanatory power for as many pathological observations as possible. The zinc cascade is merely a beginning in this context.

As the central protein of AD, APP has recently been found to possess ferroxidase function similar to ceruloplasmin that is inhibited by free  $Zn^{2+}$ ,<sup>290</sup> this provides a possible connection between the metal cascades and the amyloid cascade that could also explain the decreased transferrin levels observed in AD.<sup>388</sup> Since APP is located in the mitochondrial membranes and is a ferroxidase, APP may control iron(III) import into neuron mitochondria, perhaps in concert with APP cleavage and other functions. Zinc appears to control both this function and the  $\alpha$ -secretase turnover. Impairment of the ferroxidase function by zinc dyshomeostasis could thus suggest that amyloid imbalance is a consequence of impaired ferroxidase function in APP. Such a concept could put APP as the director of mitochondrial energy metabolism by importing iron(III) for respiration, uniting the amyloid cascade with the iron and oxidative stress cascades, as well as the zinc cascade, via the regulatory role of  $Zn^{2+}$  on both APP ferroxidase activity and  $\alpha$ -secretase activity.

### 13. CONCLUDING REMARKS: TEN FOCAL POINTS OF FUTURE RESEARCH

AD, the major form of dementia, is one of mankind's grand challenges, a devastating disease affecting millions of people and their families, and a substantial and growing burden to society, with prevalence increasing rapidly.<sup>1,3,4</sup> Because the pathogenic mechanism of AD is poorly understood, current treatments are mainly symptom-relieving and are at best effective for up to a year.<sup>18,53</sup>

The role of metals such as copper, iron, calcium, and zinc in AD has dramatically expanded over the last decades.<sup>10–12,14,17,68,144,145,147,159</sup> A large number of research groups are devoted to an understanding of the underlying biochemical causes of AD, and most of these involve pathways that are governed by regulatory or catalytic metal sites. In addition, most known environmental and lifestyle risk factors of AD can be related to underlying biochemical processes that directly rely on metal ions, as described in this review.

The role of metal dyshomeostasis is also emerging with respect to other neurological disorders, which are closely associated with metal ion-controlled structural and functional integrity of proteins such as APP, prion protein,  $\alpha$ -synuclein, and SOD-1, and in terms of AD, specifically APP,  $A\beta$ , a large range of zinc-dependent proteases involved in  $A\beta$  production and clearance, and underlying metal-homeostatic proteins, notably MTs, which have been described in this review. Because of this emerging paradigm shift, the vibrant field of bioinorganic chemistry may now have a unique opportunity to interact with the enigmatic and immensely complex research in neurological disorders. Hopefully, bioinorganic chemists will take up the challenge. This review has attempted to provide a first encounter between the fields.

Some of the most interesting focal points of future AD research on the interface between bioinorganic chemistry and neuroscience are described in the following:

1. Metal ion locations in time and space during AD pathogenesis. A clear consensus should be established regarding the free and bound pools of Zn, Cu, and Fe in various parts of the brain as a function of disease progression, to remove current uncertainties in this regard,<sup>152</sup> because the redistribution from bound to free

pools seems to be critical in disease progression. An understanding should be achieved of how these metal ion imbalances affect APP processing,  $A\beta$  production–clearance balance, and molecular toxicity at various stages of AD.

2. Risk factors and metal homeostasis. A specific focal point of mechanistic understanding is genetic, lifestyle, and environmental causes of metal ion dyshomeostasis and how these relate etiologically to other pathological features such as oxidative stress,  $A\beta$  imbalance, and tau hyperphosphorylation. Is sporadic AD a spiral etiology of all these risk factors, together increasing total risk of AD as age progresses, or are there critical thresholds and turning points? Homeostasis should be further explored, for example, via metallothioneins, prion proteins, copper and zinc transporters, iron regulatory proteins, ceruloplasmin, divalent metal transporter 1,<sup>383</sup> and heme oxygenases, with a focus on understanding how genetic weaknesses or stress-induced post-translational modifications impair the functions of these proteins.
3. APP-regulating chelators. A third focal point of future therapies could be the use of coordination chemistry expertise in the design of new  $\alpha$ -secretase enhancers and  $\beta/\gamma$ -secretase inhibitors that protect the regulatory zinc and copper sites in APP to restore the nonamyloidogenic pathway. Given the relatively large  $K_d$ 's of natural antioxidant chelators, it is plausible that EGCG, curcumin, and lipoic acid enhance  $\alpha$ -secretase activity<sup>874,892,894</sup> by stripping excess  $Zn^{2+}$  from  $\alpha$ -secretase-regulatory APP sites or bind excessive free  $Zn^{2+}$  accumulated by zinc dyshomeostasis, which inhibit APP ferroxidase activity and thus potentially cause iron dyshomeostasis and metabolic disruption in neuronal mitochondria. While some of these ideas for future AD prevention have been recently discussed,<sup>447</sup> having zinc and copper regulatory sites in APP as specific targets has not yet been pursued.
4. Metal–protein-attenuating compounds. A fourth focal point would be the clinical development of new metal chelators, which can either target the Cu(II) and Zn(II) amyloid oligomers directly to enable subsequent degradation by, for example, neprilysin, which is also Zn(II)-dependent, or target free metal ions that induce amyloidosis. Current development of chelators such as clioquinol has been somewhat disappointing, and more knowledge is required,<sup>995</sup> because metal chelation constitutes a risky perturbation of the CNS. Too strong chelators may be dangerous because they can strip active sites and normal regulatory sites of their metal ions; thus they should be designed with appropriate and selective binding in mind, with key dissociation constants for such designs discussed in this review. The history of clioquinol, notably the SMON outbreak in Japan, showed the risks associated with large doses of metal chelators. One of several toxic modes of  $A\beta$  possibly relates to its stripping of Cu from prion protein to disrupt NMDA receptors and  $Ca^{2+}$  signaling,<sup>119</sup> and possibly, the large amount of copper, iron, and zinc found in amyloid plaques derive from proteins that have lost their function during the AD pathogenesis.
5. Oligomerization inhibitors. A fifth, related focal point would be preferential stabilization of innocent, non-

oligomeric complexes involved in metal–amyloid interaction mechanisms.<sup>195</sup> Such a strategy would include development of Cu–A $\beta$  transition state (de)stabilizing molecules that either lower the barrier for formation of innocent A $\beta$ –Cu–A $\beta$  species or enlarge the barrier for the competing oligomerization steps. All approaches mentioned in 3–5 rely on the conscious design of adequate BBB-penetrable, drug-like metal chelators exhibiting  $K_d$ 's in the correct ranges (in the  $10^{-10}$  M range) with emphasis on selectivity between Cu, Zn, and Fe, depending on the target. For example, Zn(II) will often bind  $\sim 100$  times more weakly than Cu(II) in 1:1 ratios as seen in amyloids and various relevant proteins, and a balance between soft and hard ligands may aid toward obtaining optimal selectivity while preventing stripping bound Zn(II) and Cu(II) from active sites of key proteins.

6. Bioinorganic diagnosis. The bioinorganic chemistry of AD may also substantially contribute to new, efficient, and unambiguous diagnosis, given that amyloid load is a poor marker for progression, and blood protein markers may be feasible.<sup>696,997</sup> Efficient biomarkers that detect AD in the preclinical phase could markedly improve treatment.<sup>26</sup> Current markers include absolute levels of A $\beta$ 42 in the cerebrospinal fluid (decreased in AD) and overall amyloid retention in the brain (increased in AD), tau protein levels in the cerebrospinal fluid (increased in AD), structural evidence for neurodegeneration from magnetic resonance imaging, and, as seen in other neurodegenerative diseases, impaired glucose (fluoro-deoxyglucose) uptake probably due to impaired mitochondrial function.<sup>9</sup> Biomarkers may be ranked according to their diagnostic accuracy and ability to provide early diagnosis, suggesting that A $\beta$  markers rank above tau protein markers.<sup>6,9</sup> The same logic might be applied to markers of free vs bound metal ion pools. Literature examples include iron heme pools as a marker,<sup>387</sup> MT levels as a marker,<sup>600</sup> or the ratio of Cu to MT or ceruloplasmin that may monitor AD progression.<sup>622</sup> Given the findings reviewed here, one might suggest to investigate any combination of the local ratios between Fe, Zn, Cu, ceruloplasmin, heme oxygenase, and MT levels together with oxidative stress markers, APP or secretase levels, and A $\beta$ 42/A $\beta$ 40 ratios.<sup>100</sup> Ratios are likely to be less sensitive to individual variation and better reflect *de facto* dyshomeostasis and may be encouraged. Some ratios may be achieved either in blood serum, plasma, or cerebrospinal fluid, remembering that disease progress correlates with local metal redistributions from bound intracellular to free pools. Also, as mentioned in focal point 1, marker output should be a function of time as well as space.
7. Multifunctional antioxidants. A somewhat different approach is the focus on antioxidant medicine, which can however in many cases be combined with metal chelator function, as evident in a number of natural antioxidant chelators.<sup>649</sup> Also, understanding the extremely complex and wide-ranging interplay between metal homeostasis and oxidative stress in neurological disorders should probably be a primary objective in the future.
8. Clear distinctions between AD and accelerated aging. Given the similarities between various types of dementia

and geriatric depression<sup>26</sup> and the similarities in the underlying neurochemical changes, notably oxidative stress and metal ion homeostasis, a better understanding of possible critical events that trigger sporadic AD seems warranted. A central focal point could be the distinct differences between AD and “accelerated aging”, for example, by comparing AD pathological features not just to same-age controls but also to older controls. Such research would pinpoint exactly how AD distinguishes itself from an accelerated aged brain, for example, the sporadic causes of the amyloid imbalance. Notably, if a pathogenic threshold is reached, it should provide a transition in the levels of markers that correspond to accelerated aging, toward markers that are unique to AD. The timing of markers is therefore crucial. The various markers could be tested both in same-age and in older-age controls and in AD.

9. Lifestyle and diet in relation to metal homeostasis. A ninth focal point is the optimization of lifestyle and dietary prevention strategies that may reduce the nongenetic risk factors discussed in this review, which in many cases relate directly to metal ion homeostasis or oxidative stress.<sup>860</sup> Importantly, we are beginning to understand why certain diets and lifestyles are beneficial or constitute risk factors, relating to the complex interplay between metabolism, metal homeostasis, and oxidative stress, and this would contribute to our overall understanding of the bioinorganic chemistry of AD.
10. Causes of the cellular origins of AD. A tenth focal point is to identify the cellular origins of AD, where many paths seem to converge toward a role of the impaired zinc-enriched neurons in the parts of the brain most affected by AD, hippocampus and the astrocytes, which maintain neuron (metal) homeostasis and are particularly rich in metal ions and MTs. To exemplify this, aceruloplasminemia, the neurological disease associated with mutations in the multicopper ferroxidase ceruloplasmin, leads to deformation of astrocytes,<sup>998</sup> and MT-3 can reduce neurodegeneration in mouse hippocampus.<sup>605</sup>

As will hopefully be clear from reading this review, bioinorganic chemistry and neuroscience, while previously considered somewhat incommensurable, will continue to produce tremendous synergies in the future. The main goal of such an alliance would probably be new combination treatments that address in a systemic way several of the risk factors and imbalances described in this review, for example, combinations of MPACs, antioxidants, and APP modulators. Particular emphasis might be placed on combining chelators with affinities for Fe, Cu, and Zn suitable for APP modulation and inhibition of A $\beta$  oligomerization with simultaneous antioxidant function. Such combination treatments could not only reduce side effects but in fact be necessary, because other treatments addressing only one aspect of the etiology will most likely be inefficient. Thus, the impact of the bioinorganic chemistry with a strong emphasis on structure–function correlations, careful identification of key targets, and a systemic approach to underlying pathways as attempted here could be game-changing and may hopefully help to improve the prospects for the millions of people suffering from this terrible disease.



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The authors declare no competing financial interest.

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## LIST OF ABBREVIATIONS AND ACRONYMS

|           |  |
|-----------|--|
| A $\beta$ | amyloid- $\beta$ , 40- or 42-residue peptide derived from APP; the most characteristic molecular symptom of AD           |
| AD        | Alzheimer's disease  |
| ALS       | amyotrophic lateral sclerosis  |
| ADAM      | a disintegrin and metalloprotease family. Family of proteins that include zinc proteases of the $\alpha$ -secretase type |
| ApoE      | apolipoprotein E   |
| ApoE4     | isoform 4 of ApoE  |
| APP       | amyloid precursor protein  |
| ASD       | autism spectrum disorder   |
| BACE-1    | another acronym for $\beta$ -secretase (beta-site APP cleaving enzyme 1)   |
| BBB       | blood-brain barrier  |
| CNS       | central nervous system   |
| Ctr1      | copper transport protein 1   |
| DMT-1     | divalent metal transporter 1, an iron and copper transport protein active in neurons                                     |
| GABA      | $\gamma$ -aminobutyric acid, the main inhibitory neurotransmitter of the CNS   |
| GSH       | glutathione; an important antioxidant  |

|               |   |
|---------------|---|
| GSSG          | glutathione disulfide; the oxidized dimer of GSH containing a disulfide bridge  |
| HIF           | hypoxia-inducible factor; transcription factor activated by hypoxia or lack of Fe and governing a large part of the cellular response to energy input shortage-related stress |
| HO-2          | heme oxygenase isoform 2, a heme degrading enzyme expressed in neurons  |
| LRP1          | lipoprotein-receptor-related protein 1, aka apolipoprotein E receptor (APOER)   |
| MCI           | mild cognitive impairment   |
| MES           | methionine synthase, a cobalt (B <sub>12</sub> ) enzyme converting homocysteine to methionine of importance in neurochemistry   |
| MPAC          | metal-protein-attenuating compounds   |
| MT            | metallothionein; there are four main classes, MT-1, MT-2, MT-3, and MT-4; MT-1 has many isoforms  |
| MTF-1         | metal-responsive transcription factor-1   |
| NMDA          | N-methyl-D-aspartate, an amino acid neurotransmitter agonist working on glutamate-dependent receptors of the NMDA-R type  |
| NMDA-R        | N-methyl-D-aspartate receptor, a class of glutamate receptors that are affected by NMDA   |
| RNS           | reactive nitrogen species, for example, nitric oxide, NO <sup>•</sup>   |
| ROS           | reactive oxygen species, for example, H <sub>2</sub> O <sub>2</sub> , O <sub>2</sub> <sup>-</sup> , HO <sup>•</sup>   |
| SAM           | S-adenosyl methionine, a central molecule in the methyl transfer system (the SAM Cycle) and in neurochemistry; AD is associated with low SAM levels                           |
| sAPP $\alpha$ | secreted, soluble amyloid precursor protein, a nonamyloidogenic peptide fragment derived from APP by cleavage of the $\alpha$ -site by $\alpha$ -secretase                    |
| SOD           | superoxide dismutase; there are three forms in humans, intracellular SOD-1, containing Cu and Zn, SOD-2, containing Fe or Mn, and extracellular SOD-3, containing Cu and Zn   |
| STAT3         | signal transducer and activator of transcription 3; transcription factor involved in cell growth and apoptosis  |
| ZEN           | zinc-enriched neurons; glutamatergic neurons that co-release Zn <sup>2+</sup> from synaptic vesicles to modulate neurotransmission  |
| ZIP           | a family of zinc-transporter proteins, important for controlling the free Zn <sup>2+</sup> pool   |
| ZnT           | a family of zinc-transporter proteins; transports Zn <sup>2+</sup> into synaptic vesicles of ZEN  |

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