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## Therapeutics for Alzheimer's Disease Based on the Metal Hypothesis

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### SUMMARY

Alzheimer's disease (AD), the most common form of dementia in the elderly, is characterized by elevated brain iron levels and accumulation of copper and zinc in cerebral  $\beta$ -amyloid deposits; e.g., senile plaques. Both ionic zinc and copper are able to accelerate the aggregation of A $\beta$ , the principle component of  $\beta$ -amyloid deposits. Copper (and iron) can also promote the neurotoxic redox activity of A $\beta$  and induce oxidative cross-linking of the peptide into stable oligomers. Recent reports have documented the release of A $\beta$  together with ionic zinc and copper in cortical glutamatergic synapses following excitation. This, in turn, leads to the formation of A $\beta$  oligomers, which, in turn, modulate long-term potentiation (by controlling synaptic levels of the NMDA receptor). The excessive accumulation of A $\beta$  oligomers in the synaptic cleft would then be predicted to adversely affect synaptic neurotransmission. Based on these findings, we have proposed the "Metal Hypothesis of Alzheimer's Disease" which stipulates that the neuropathogenic effects of A $\beta$  in AD are promoted by, and possibly even dependent upon A $\beta$ -metal interactions. Increasingly sophisticated pharmaceutical approaches are now being implemented to attenuate abnormal A $\beta$ -metal interactions without causing systemic disturbance of essential metals. Small molecules targeting A $\beta$ -metal interactions, e.g. PBT2, are currently advancing through clinical trials and show increasing promise as disease-modifying agents for AD based on the "metal hypothesis".

### Keywords

copper; zinc; amyloid; free radical; oxidation; PBT2

### 1. Introduction

Currently, the most popular hypothesis for Alzheimer's disease (AD)-related cognitive dysfunction and neuropathogenesis is the "Amyloid Cascade Hypothesis", which posits that all pathology in the AD brain occurs downstream of the excessive accumulation of  $\beta$ -amyloid

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in the CNS.<sup>1,2</sup> However, while a central role for A $\beta$  in the pathogenesis of AD is indisputable based largely on genetics, considerable evidence indicates that A $\beta$  production is not the sole culprit in AD pathogenesis.<sup>2</sup> This problem is central to the ability to develop disease-modifying therapies for AD. Currently-marketed drug therapies for AD target symptom relief, but do not interdict the underlying causal pathobiology. However, other more recent approaches to drug development for AD have been targeted at curbing disease progression. Within this realm, the greatest emphasis has been placed on blocking  $\beta$ -amyloid accumulation, e.g. senile plaques, in the brain. Genetic studies clearly implicate alterations in A $\beta$  production in the pathogenesis of AD,<sup>2</sup> however, it remains unclear as to how A $\beta$  accumulates in brain and leads to cognitive dysfunction and dementia. Moreover, while A $\beta$  is neurotoxic at non-physiological (micromolar) concentrations *in vitro*, it is normally produced in the brain,<sup>3</sup> and at physiological (nanomolar) concentrations, has even been shown to possess neurotrophic properties in cell culture.<sup>4–6</sup> Thus, in targeting A $\beta$  for the treatment of AD, other factors influencing A $\beta$  toxicity must also be elucidated and pharmaceutically addressed.

The length of A $\beta$  is an important factor in AD pathogenesis; the less prevalent form of the peptide, A $\beta$ 42,<sup>7</sup> is particularly enriched in  $\beta$ -amyloid deposits.<sup>8,9</sup> Furthermore, most of the ~200 known early-onset familial AD-linked mutations in the amyloid precursor protein (APP) and presenilin genes do not increase overall production of A $\beta$ , but, instead, increase the ratio of A $\beta$ 42:A $\beta$ 40.<sup>2,10</sup> Synthetic A $\beta$ 42 aggregates more readily than A $\beta$ 40 and A $\beta$ 42 readily seeds the aggregation of A $\beta$ 40, *in vitro*.<sup>11,12</sup> Aggregated A $\beta$ , e.g. in the form of soluble oligomers, has been implicated as the neurotoxic form of the peptide.<sup>2,13–15</sup> These pathophysical properties of A $\beta$  form the basis of the amyloid cascade hypothesis, which maintains that excessive production of A $\beta$  is sufficient to cause of AD. The problem with applying this hypothesis to most forms of AD is that the self-aggregating properties of A $\beta$  alone are insufficient to explain the accumulation of the peptide in specific brain regions of AD patients. Healthy people normally have soluble A $\beta$  in their brains, and A $\beta$  is a soluble component of all biological fluids. Therefore, it is conceivable that there may be an abnormally modified “rogue” form of soluble A $\beta$  that is particularly neurotoxic in AD. In this review, we will propose that it is the interaction of A $\beta$  with specific metals, particularly copper and zinc, which drives A $\beta$  pathogenicity and downstream AD pathology. We have coined this “The Metal Hypothesis of Alzheimer’s Disease”.

Based upon the overwhelming genetic and pathophysiological evidence supporting A $\beta$  as the culprit molecule in AD, the major approaches for developing therapeutics to slow, stop, or reverse AD progression have attempted to either target A $\beta$  production (e.g., secretase inhibitors and modulators), or to clear A $\beta$  from the brain (e.g., immunotherapy). However, other neurochemical events apart from A $\beta$  production may also contribute to  $\beta$ -amyloid deposition and toxicity in AD. Were elevated cortical A $\beta$  concentrations to be solely responsible for the deposition of  $\beta$ -amyloid, it would be difficult to explain why  $\beta$ -amyloid deposits are focal (related to synapses, and the cerebrovascular lamina media) and not uniform in their distribution, especially since APP and A $\beta$  are ubiquitously expressed. Moreover, to attribute  $\beta$ -amyloid accumulation to the presence of A $\beta$ 42, alone, is problematic since the peptide is a normal component of healthy CSF.<sup>7</sup> Finally, while  $\beta$ -amyloid deposition is an age-dependent phenomenon, A $\beta$  production does not appear to increase with age. Thus, other age-related stochastic changes, e.g. metal-mediated oxidative damage to neuronal cells, which generally precedes A $\beta$  deposition,<sup>16–18</sup> most likely play essential roles in the biochemical events and reactions that cause A $\beta$  to accumulate in specific brain regions affected in AD.

## 2. Metallochemistry mediates the aggregation and neurotoxicity of A $\beta$

We first discovered in 1994 that A $\beta$  becomes amyloidogenic upon reaction with stoichiometric amounts of Zn<sup>2+</sup> and Cu<sup>2+</sup>.<sup>19,20</sup> In the subsequent years it has become clear that A $\beta$  is a

metalloprotein,<sup>21,22</sup> and that the brain's intrinsic supply of  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  (and possibly  $\text{Fe}^{3+}$ ) mediates the peptide's toxicity (through radical and hydrogen peroxide production) and aggregation. We first observed that  $\text{A}\beta$  is rapidly precipitated by  $\text{Zn}^{2+}$ .<sup>19,20,23</sup>  $\text{Cu}^{2+}$  and  $\text{Fe}^{3+}$  also induce marked  $\text{A}\beta$  aggregation but only under mildly acidic conditions (e.g., pH 6.8--7.0),<sup>19,20,23</sup> such as those in the brains of AD patients.  $\text{Cu}^{2+}$  precipitates  $\text{A}\beta$  more robustly than  $\text{Fe}^{3+}$ , and even trace (nanomolar) concentrations of  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$  or  $\text{Fe}^{3+}$  in common laboratory buffers are sufficient to induce nucleation of  $\text{A}\beta$ , which can then lead to fibrillization of the peptide solution.<sup>24–26</sup> Interestingly, rat and mouse  $\text{A}\beta$  possesses amino acid substitutions that decrease metal interactions,<sup>20</sup> perhaps explaining why these animals are exceptional among mammals for not accumulating cerebral  $\text{A}\beta$  amyloid with advanced age.<sup>27</sup> On the basis of our findings regarding  $\text{A}\beta$ -metal interactions, in 1997, we co-founded the company, Prana Biotechnology, Ltd., which has since initiated a clinical program focusing on a new class of drug therapy targeting  $\text{A}\beta$ -metal biochemistry (see below).

$\text{A}\beta$  possesses selective high and low affinity metal binding sites, which are histidine mediated.<sup>25,28,29</sup> The original reported  $K_d$  of high affinity  $\text{Zn}^{2+}$  binding was  $\approx 100$  nM, and for low affinity binding  $\approx 5$   $\mu\text{M}$ .<sup>19,20</sup> While there has been some contention about the exact  $K_d$  values for both  $\text{Zn}^{2+}$  and  $\text{Cu}^{2+}$ , it is now understood that both the buffer conditions (e.g., the presence of  $\text{NaCl}$ <sup>30</sup>), the aggregation state of the peptide,<sup>19,31,32</sup> and the means used for assaying the bound and free metal ions<sup>33</sup> are critical for the observed values. However, a consensus has emerged that the  $\mu\text{M}$  concentrations of both  $\text{Zn}^{2+}$  and  $\text{Cu}^{2+}$  that are released from cortical synapses are sufficient to induce  $\text{A}\beta$  aggregation.<sup>23,32–34</sup> Low affinity  $\text{Zn}^{2+}$  binding mediates the precipitation of the peptide, as well as its resistance to tryptic (alpha secretase-like) cleavage.<sup>19</sup>  $\text{A}\beta$  also possesses high and low affinity  $\text{Cu}^{2+}$  binding sites.<sup>24,25</sup> Although the affinity of the low affinity  $\text{Cu}^{2+}$  binding site is similar between  $\text{A}\beta 1-40$  and  $\text{A}\beta 1-42$  ( $5.0 \times 10^{-9}$  M), the affinity of the high affinity site on  $\text{A}\beta 1-42$  has been reported as  $7.0 \times 10^{-18}$  M, which may be the product of a perturbed equilibrium caused by precipitated  $\text{A}\beta$  withdrawing  $\text{Cu}^{2+}$  from solution. This is much greater than the highest observed affinity of  $\text{A}\beta 1-40$  for  $\text{Cu}^{2+}$  ( $5.0 \times 10^{-11}$  M).<sup>25</sup> The higher affinity of  $\text{A}\beta 1-42$  versus  $\text{A}\beta 1-40$  for  $\text{Cu}^{2+}$  nicely correlates with enhanced precipitation of  $\text{A}\beta 1-42$  by  $\text{Cu}^{2+}$ ,<sup>24, 25</sup> increased SDS-resistant dimerization of  $\text{A}\beta 1-42$  by  $\text{Cu}^{2+}$ ,<sup>24</sup> and the increased redox activity of the  $\text{Cu}^{2+}:\text{A}\beta 1-42$  complex (see below).

$\text{A}\beta$  binds equimolar amounts of  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  at pH 7.4. However, under conditions representing acidosis (pH 6.6)  $\text{Cu}^{2+}$  completely displaces  $\text{Zn}^{2+}$  from  $\text{A}\beta$ .<sup>25</sup>  $\text{A}\beta$  binds up to 2.5 equivalents of either  $\text{Cu}^{2+}$  or  $\text{Zn}^{2+}$ , the fractional stoichiometry indicating that metal binding is possibly coordinated by oligomers.<sup>25</sup> This would have implications for utilizing hexafluoroisopropanol, commonly employed for monomerizing  $\text{A}\beta$  *in vitro*. The positive cooperativity in  $\text{Cu}^{2+}$  binding observed for  $\text{A}\beta$  may be greater for  $\text{A}\beta 1-42$  than for  $\text{A}\beta 1-40$  because of the enhanced ability of the longer peptide to form a  $\text{Cu}^{2+}$  coordinating oligomer.<sup>35</sup> Intriguingly, Apolipoprotein E (ApoE) isoforms prevent copper-mediated aggregation of  $\text{A}\beta$  in a manner that correlates with their risk for AD,<sup>36</sup> and the precipitation of  $\text{A}\beta$  by  $\text{Zn}^{2+}$  and  $\text{Cu}^{2+}$  is reversible with chelation,<sup>24,30,37,38</sup> in contrast with fibrillization, which is irreversible.

Beyond assembling  $\text{A}\beta$  into oligomers and fibrils, upon binding  $\text{Cu}^{2+}$  or  $\text{Fe}^{3+}$ ,  $\text{A}\beta$  reduces these metal ions and produces  $\text{H}_2\text{O}_2$  by double electron transfer to  $\text{O}_2$  (there is no evidence of  $\text{O}_2^-$  formation as an intermediate),<sup>21</sup> a reaction that has since been repeatedly confirmed.<sup>39–41</sup> This electrochemistry, which is critical for  $\text{A}\beta$ -induced oxidative stress and toxicity in cell culture, is partly mediated by methionine<sup>35,42,43</sup> and tyrosine<sup>10,44</sup>.  $\text{H}_2\text{O}_2$  is also formed catalytically by the cycling of copper or iron bound to  $\text{A}\beta$  using biological reducing agents as electron donors without net oxidation of the  $\text{A}\beta$  peptide. The most likely electron donors pathophysiologically are cholesterol and long-chain fatty acids,<sup>21,41,45–48</sup> consistent with

the toxicity of A $\beta$  being mediated by adherence to the cell membrane<sup>42</sup> and the consequent production of toxic lipid oxidation products oxysterols and 4-hydroxynonenal (HNE), which are elevated in affected brain tissue in AD and in APP transgenic mice.<sup>21,41,45,46,48</sup> A $\beta$  promotes copper-mediated generation of HNE from polyunsaturated lipids, and in turn, HNE covalently modifies the histidine side chains of A $\beta$ .<sup>49</sup> HNE-modified A $\beta$  has an increased affinity for lipid membranes and an increased tendency to aggregate into amyloid fibrils.<sup>49</sup> Thus, the pro-oxidant activity of A $\beta$  ultimately leads to its own covalent modification and accelerated amyloidogenesis. It should be noted that catecholamines can also be oxidized by A $\beta$ :Cu complexes.<sup>21,50,51</sup>

These reactions are important because there is overwhelming evidence in the literature for oxidative injury in AD, mediated by H<sub>2</sub>O<sub>2</sub>. H<sub>2</sub>O<sub>2</sub> is a pro-oxidant molecule that is the substrate for the Fenton reaction that generates the highly reactive hydroxyl radical (OH•). H<sub>2</sub>O<sub>2</sub> is freely permeable across all tissue boundaries and will react with reduced metal ions (Fe<sup>2+</sup>, Cu<sup>+</sup>) to generate OH•, which in turn, generates lipid peroxidation adducts, protein carbonyl modifications, and nucleic acid adducts such as 8-OH guanosine, in all cellular compartments, which typify AD neuropathology.<sup>52–54</sup> In AD, the H<sub>2</sub>O<sub>2</sub> scavenging defenses, e.g. catalase and glutathione peroxidase, may be overwhelmed by the catalytic generation of H<sub>2</sub>O<sub>2</sub> from the A $\beta$  metalloprotein mass. The redox activity (metal reduction, OH• and H<sub>2</sub>O<sub>2</sub> formation) of A $\beta$  variants is greatest for A $\beta$ <sub>42</sub><sub>human</sub> > A $\beta$ <sub>40</sub><sub>human</sub> >> A $\beta$ <sub>40</sub><sub>mouse</sub>  $\approx$  0.<sup>55</sup> This rank order is strikingly relevant to AD pathogenesis since A $\beta$ <sub>42</sub> production is enhanced by fully penetrant, early-onset familial AD mutations in APP and the presenilin genes, and A $\beta$ <sub>42</sub> is considerably more prone to aggregation into neurotoxic assemblies (*versus* A $\beta$ <sub>40</sub> and rodent A $\beta$ ). This redox relationship also corresponds to the neurotoxicity of the respective peptide in neuronal culture, which is largely mediated by the Cu<sup>2+</sup>:A $\beta$  interaction.<sup>21,55</sup> Notably, the interaction of A $\beta$  with the cell membrane is promoted by binding Cu<sup>2+</sup> and Zn<sup>2+</sup>.<sup>35,56</sup> Conversely, copper- and iron-chelators like TETA block these electrochemical reactions and attenuate A $\beta$  toxicity in cell culture.<sup>45,57</sup>

A $\beta$  coordination of copper leads to the generation of ROS involving the reduction of the oxidation state of the coordinated Cu<sup>2+</sup> to Cu<sup>1+</sup>. When this reduction reaction is not accompanied by the oxidation of another moiety, such as cholesterol,<sup>45</sup> A $\beta$  side-chains can become oxidized. This can then lead to a variety of oxidized A $\beta$  species, as well as cross-linking of A $\beta$  peptides. Mass spectrometry has shown that Cu<sup>2+</sup> ions are able to oxygenate A $\beta$ , with the most likely target being the sulphur atom of methionine 35 (Met<sub>35</sub>).<sup>43</sup> In addition to A $\beta$  methionine sulfoxide, a number of other adducts can be generated from copper-mediated redox reactions including aldehyde adducts to the lysine residues<sup>58</sup> and tyrosine modified with adducts such as DOPA, dopamine, dopamine quinone, dihydroxyindol and isodityrosine.<sup>43, 59</sup> 2-oxo-histidine adducts of A $\beta$  have also been extracted from AD plaques;<sup>60</sup> N3-pyroglutamate modified forms of A $\beta$  are the main ligands for the amyloid PET ligand PIB.<sup>61</sup> Tyrosine is particularly susceptible to free radical attack due to its conjugated aromatic ring. Elevated levels of dityrosine and 3-nitrotyrosine have been reported within neuronal lesions in AD brain. In the presence of Cu<sup>2+</sup> and H<sub>2</sub>O<sub>2</sub>, A $\beta$ <sub>42</sub> forms dityrosine cross-linked oligomers *in vitro*, a modification that is resistant to proteolysis.<sup>62</sup> The formation of dityrosine cross-linked A $\beta$  further facilitates aggregation, leading to higher order oligomers.<sup>44</sup> A $\beta$ -generated radicals formed after reduction of copper can also form covalent adducts onto other proteins. Along these lines, peroxidases like cyclooxygenase 2 (COX2) are particularly vulnerable because of the formation of dityrosine bridges, and we have previously reported that levels of COX2-A $\beta$  covalent complexes are elevated in AD brain.<sup>63</sup>

While a wide and diverse array of hypotheses have been proposed for the mechanism by which A $\beta$  induces its neurotoxic effects,<sup>2</sup> there is general agreement that aggregation of A $\beta$  is required. Neurotoxicity has been reported for virtually every aggregate of A $\beta$  tested, from

dimers to mature fibrils. Along these lines, over the past several years, there has been increasing interest in soluble A $\beta$  oligomers, which appear to be particularly toxic.<sup>64</sup> With regard to A $\beta$ -metal interactions, covalent cross-linking of A $\beta$  (e.g., dityrosine formation generated by copper oxidation) could conceivably contribute greatly to the formation of toxic soluble A $\beta$  species.<sup>44</sup> Interactions of A $\beta$  with metal ions may also explain the increased involvement of soluble oligomeric species of A $\beta$  in AD pathogenesis. Zn<sup>2+</sup> and Cu<sup>2+</sup> readily precipitate A $\beta$  oligomers.<sup>19,31</sup> One recent report has shown that the N-terminal region of A $\beta$  can access a range of metal-coordination structures, and that A $\beta$ -Cu<sup>2+</sup> coordination correlates with peptide self-assembly and neurotoxicity.<sup>65</sup>

### Zinc and copper are the critical for A $\beta$ aggregation in AD brain

Zinc, copper, and iron have been shown in multiple studies to be markedly enriched in A $\beta$  deposits (plaques and congophilic angiopathy) in AD patients and in AD transgenic mice.<sup>22, 66-72</sup> Copper (390 $\mu$ M), zinc (1055 $\mu$ M) and iron (940 $\mu$ M) have been reported to be elevated by several-fold in AD brain as compared to normal age-matched samples (copper (70 $\mu$ M), zinc (350 $\mu$ M) and iron (340 $\mu$ M)).<sup>66</sup> A $\beta$  directly coordinates copper and zinc, but not iron or other metal ions, within the cores of plaques.<sup>21,22</sup> Iron is found in the plaque periphery, but primarily complexed with ferritin in the neuritic component of plaques.<sup>73</sup> Iron is also found together with copper and zinc within neurons and NFTs.<sup>74-76</sup> Consistent with a role for A $\beta$ -metal interaction in AD pathogenesis, experiments in ZnT3 knockout mice have established that presynaptic zinc release leads to  $\beta$ -amyloid formation in mutant APP transgenic mice. Moreover, genetic ablation of ZnT3 markedly inhibited  $\beta$ -amyloid pathology and congophilic angiopathy<sup>69,77</sup> in these mice, increasing the concentration of soluble A $\beta$ .<sup>77</sup> These findings suggest that soluble A $\beta$  and soluble zinc exist in a dissociable equilibrium with insoluble plaque A $\beta$  (containing incarcerated zinc), a process we have previously termed the "galvanization" of amyloid.<sup>78</sup> Increased amyloid deposition in female mutant APP transgenic mice also may be explained by estrogen-dependent increases in ZnT3 expression.<sup>79</sup>

Over the past decade, we have followed up on our initial findings on the contribution of A $\beta$ -metal interactions to AD pathogenesis by developing novel therapeutic approaches aimed at interfering with A $\beta$ -metal binding. Initially, for purposes of proof of concept, we showed that Zn/Cu-selective chelators could prevent A $\beta$  aggregation *in vitro*, and markedly enhance the resolubilization of A $\beta$  deposits from post-mortem AD brain samples.<sup>80</sup> The observed increase in extractable A $\beta$  from *postmortem* human brain specimens correlated with significant depletion in zinc (30%) and to a lesser extent, copper.<sup>80</sup> The ability of chelators to extract A $\beta$  depended upon the presence of Mg<sup>2+</sup> and Ca<sup>2+</sup>, hence the chelating compound needed to be far more selective for Zn<sup>2+</sup> and Cu<sup>2+</sup>, than Ca<sup>2+</sup> and Mg<sup>2+</sup>.<sup>80</sup> These results fostered the first generation of attempts to target A $\beta$ -metal interactions with the goal of inhibiting amyloid pathology in APP transgenic mice, which are discussed later in this review.

### The neurochemistry of transition metal ions in the cortex and glutamatergic synapse

A common misconception in discussions of the pathological mechanism underlying neurological syndromes where abnormal metal homeostasis has been implicated is that neurodegeneration is brought on by toxicological exposure to Cu, Fe, Zn and Mn. In other words, ingestion or enhanced exposure to the metals purportedly causes abnormal protein interactions, which then causes the disease. This is an important misconception to clarify. In terms of total metal concentrations, the brain has more than enough of these metal ions resident in its tissue to damage or corrupt the activities of numerous proteins and biochemical pathways. For example, the concentration of Zn<sup>2+</sup> that is released during neurotransmission is  $\approx$ 300  $\mu$ M, which is more than sufficient to be rapidly neurotoxic in neuronal cell culture.<sup>81</sup> Therefore, the brain must possess efficient homeostatic mechanisms and buffers in place to prevent the



abnormal decompartmentalization of metal ions. It should also be noted that the blood-brain barrier (BBB) is relatively impermeable to fluctuating levels of plasma metal ions.

Generally, in health, biometals, iron, copper, zinc are bound to ligands (e.g., transferrin) and not found as free species. However, recent data have documented the release of free ionic or exchangeable zinc and copper in the synaptic cleft (see below) upon glutamatergic excitation. Consequently, zinc is also being increasingly understood to mimic calcium as a new class of second messenger.<sup>82</sup> Furthermore, the intracellular pool of free iron, the labile iron pool, has been shown to modulate the expression of various proteins, including the amyloid protein precursor (APP).<sup>83</sup> Intracellular copper is considered to largely ligand-bound. However, it has also been shown to be exchangeable and transferred from protein to protein (e.g., by the copper chaperone of superoxide dismutase 1, CCS1).<sup>84</sup>

In the last few years, there have been several important basic discoveries about copper and zinc release and flux at the glutamatergic synapse in the cortex and hippocampus. This synapse is important not just because it is a site of long-term potentiation, but also because it is here that  $\beta$ -amyloid first deposits in AD and is one of the most important sites for cognitive dysfunction in this disorder.<sup>85</sup> There has been interest in the presence of zinc and copper released by hippocampal tissue for at least two decades.<sup>82</sup> Considerable evidence has supported the release of zinc either as a free or an exchangeable ionic species into the extracellular space.<sup>86</sup> This pool of vesicular zinc is modulated by the activity of ZnT3, which is found in the membrane of glutamatergic vesicles, but not elsewhere. This pool zinc, released together with A $\beta$  during neurotransmission, also appears to suppress long-term potentiation by attenuating synaptic levels of the NMDA receptor.<sup>87</sup> Post-synaptic NMDA neurites have also been reported to release free ionic Cu upon NMDA activation.<sup>88</sup> Activation of synaptic NMDA receptors in hippocampal neurons results in trafficking of Menkes ATPase and an associated efflux of copper.<sup>88</sup> Catalytic amounts of copper can function as electron acceptors promoting the reaction of nitric oxide with thiols. Thus it is conceivable that the release of Cu could function as a molecular switch to control extracellular S-nitrosylation of the NMDA receptor, a post-translational mechanism shown to be critical for modulating receptor function.<sup>88</sup> Copper has also been reported to be specifically protective against NMDA-mediated excitotoxic cell death in primary hippocampal neurons. This protective effect of copper depends on endogenous nitric oxide production in hippocampal neurons.<sup>89</sup> Further support for a role of copper in neurotransmission, Menkes ATPase expression is developmentally regulated, peaking during synaptogenesis, and playing a role in the endothelial cells of the BBB.<sup>90,91</sup>

Collectively, the emerging literature has described the glutamatergic synapse to be the site of an extraordinary confluence of chemically exchangeable Zn and Cu (Figure 1), which, to our knowledge, is unique in the body. This may be an explanation for how A $\beta$ , with its penchant for metal-induced precipitation and cross-linking, initially precipitates in this site in AD. It has also become clear over recent years that A $\beta$  oligomers can impair LTP by promoting the endocytosis of NMDA and AMPA receptors.<sup>87</sup> Thus, the coincident release of A $\beta$  and metals that can induce oligomerization in the synapse upon glutamatergic excitation may represent a natural means for regulating LTP. However, an excess of A $\beta$  oligomers could also pathologically impair neurotransmission in a “gain-of-function” of the same events.<sup>87</sup> One final component in this vicinity that could modulate the availability of Zn and Cu ions in the synapses is the release of metallothionein-3 (MT3 or growth inhibitory factor [GIF]) by the neighboring astrocytes,<sup>92</sup> which is decreased in AD<sup>93</sup> (Figure 1).

### Abnormal metal homeostasis in the aging brain and in AD

The metal ion content of the brain is stringently regulated with virtually no passive flux of metals from the circulation to the brain: movement of metals across the BBB is highly regulated. While iron, copper, and zinc are being increasingly implicated in interactions with

the major protein components of neurodegenerative disease, this is not merely due to increased (e.g., toxicological) levels of exposure to metals, but rather due to a breakdown in the homeostatic mechanisms that compartmentalize and regulate these metals.

The dominant risk factor associated with most neurodegenerative diseases is increasing age. Several studies in animals and humans have reported a rise in the levels of brain copper from youth to adulthood.<sup>94</sup> However, from middle age onwards, biologically available copper levels drop markedly, and are accompanied by a loss of copper-dependent enzyme activities, e.g., cytochrome c oxidase, superoxide dismutase 1, ceruloplasmin.<sup>95</sup> Age-related increases in brain iron have been documented in all species examined.<sup>96,97</sup> Indeed failure of ubiquitous ferroxidases ceruloplasmin, ferritin<sup>98</sup> and frataxin<sup>99</sup> cause neurodegenerative diseases, underscoring the vulnerability of the brain to abnormal iron regulation.<sup>100</sup> We hypothesize that the breakdown in metal ion regulation in the glutamatergic synapse, possibly inhibition of reuptake, raises the average concentrations of zinc and copper in the cleft leading to excessive A $\beta$  oligomerization and synaptotoxicity. Therefore, this has been the prime target for our pharmacotherapeutic approach (see below).

An interesting feature of the mechanism of increased AD pathology in *sod*<sup>+/-</sup> x APP transgenic mice is that brain copper, zinc and iron levels are decreased by the mitochondrial lesion.<sup>101</sup> This recapitulates a feature of the pathology of AD, where Cu levels decrease with advanced pathology.<sup>95</sup> Both dietary and genetics manipulations that increase brain Cu levels improved amyloid pathology in two strains of APP transgenic mice.<sup>102, 103</sup> However, there are also reports that exposure to copper in combination with a high fat diet increases the risk for AD;<sup>104</sup> a possibility that has drawn support in studies of rabbits exposed to copper and cholesterol.<sup>105,106</sup> In contrast, Zn levels increase in advanced AD, correlating with brain A $\beta$  burden in humans but not APP transgenic mice.<sup>95</sup> Zn nutritional deficiency is common in advanced age, and a recent report indicated that Zn deficiency in APP transgenic mice increased the volume of amyloid plaques.<sup>72</sup> These data indicate the complexity of the disordered metal metabolism in AD. The consensus that has emerged is that zinc and copper are enriched in amyloid where they coordinate A $\beta$ , iron is enriched in the tissue and neuritic pathology, and there is evidence of functional copper deficiency. Therefore, pharmacotherapy that targets abnormal A $\beta$  metallation is best geared to be not merely a chelation approach. Ideally the drug should release the metals trapped by A $\beta$ , and return them to normal metabolism, hence our interest in ionophores.

### Physiological interactions of APP and its processing with zinc and copper

While the function of APP is unknown recent evidence suggests it has a role to play in maintaining copper homeostasis.<sup>107-110</sup> APP coordinates Cu<sup>+</sup> at its amino-terminus, and APP expression promotes the export of neuronal copper.<sup>108</sup> A functional role for APP in copper homeostasis is supported by reports that cellular copper drives the expression of APP mRNA.<sup>109,110</sup>

BACE1 (beta-secretase) possesses a Cu<sup>+</sup> binding site in its C-terminal cytoplasmic domain through which it interacts with domain 1 of the copper chaperone of SOD1 (CCS1).<sup>111</sup> The functional implications of this interaction are unknown but imply that copper levels can impact upon A $\beta$  generation. Similarly,  $\gamma$ -secretase activity recently has been reported to be inhibited by low concentrations of Zn<sup>2+</sup>, however, the physiological implications are unclear.<sup>112</sup>

Abnormal brain copper distribution has been reported in AD with excessive accumulation of copper in amyloid plaques and a deficiency of copper in neighboring cells. *In vitro*, excess copper has been reported to inhibit A $\beta$  production from APP-transfected CHO cells,<sup>113</sup> however, the effects of deficiency were not previously explored. A recent report assessed the effects of modulating intracellular copper levels upon the processing of the amyloid precursor

protein and the production of  $A\beta$ .<sup>114</sup> Human fibroblasts genetically disposed to copper accumulation secreted higher levels of sAPP $\alpha$  into their medium, while fibroblasts genetically manipulated to be profoundly copper deficient secreted predominantly sAPP $\beta$  and produced more amyloidogenic C-termini (C99). Copper deficiency also markedly reduced the steady-state levels of APP mRNA while APP protein levels remained constant; indicating that copper deficiency may accelerate APP translation.<sup>114</sup> Copper deficiency in human neuroblastoma cells significantly increased the level of  $A\beta$  secretion, but did not affect the cleavage of the amyloid precursor protein.<sup>114</sup>

Several enzymes that degrade  $A\beta$  in the extracellular milieu are zinc metalloproteinases, such as neprilysin, insulin degrading enzyme, and matrix metalloproteinases<sup>94</sup>. This may explain why there is an inverse correlation between CSF zinc and copper levels and CSF  $A\beta$ <sub>42</sub> levels in normal men.<sup>115</sup> This possibility was supported by the observation that adding low micromolar concentrations of zinc or copper to ex-vivo CSF samples accelerated the degradation of  $A\beta$ .<sup>115</sup> Collectively, these data have led us to propose that  $A\beta$ -metal complexes likely play a key pathophysiological role in AD at multiple levels, supporting the “metal theory of Alzheimer’s disease”. This hypothesis of AD pathogenesis has several advantages in explaining key missing components the amyloid cascade hypothesis (Table 1). Namely, metal-mediated effects on APP and  $A\beta$  aggregation, particularly in synapses, can explain the lack of correlation of senile plaques with degree of dementia. This hypothesis is also consistent with the synaptic  $A\beta$  hypothesis,<sup>87</sup> in which metal-driven formation of  $A\beta$  oligomers in synapses can impair long-term potentiation and neurotransmission. Accordingly, over the past decade, we have worked together with Prana Biotechnology to develop drugs that can block metal-mediated  $A\beta$  reactions as a pharmacological intervention for the treatment and prevention of AD.

### Therapeutic inventions based on the metal theory of AD

Another common misconception in envisaging therapeutic approaches to AD based on the metal hypothesis is the erroneous belief that chelation, meaning the removal of metal ions from tissue, is the obvious intervention. While there are several medical chelators, their approved use is confined to genuine situations of metal overexposure (e.g., Wilson’s disease, or lead toxicity), or rheumatoid arthritis. The risk of chelation therapy is that the removal of essential metal ions will lead to serious adverse effects; e.g., iron-deficiency anemia. While these effects can, to some extent, be potentially mitigated by engineering small molecules to target specific compartments or organelles, sequestration of metals in plaques and their relative deficiency within neighbouring cells, dictates the need for the development of small molecules with more sophisticated properties; e.g., metal-protein attenuation compounds (MPAC) that serve as metal exchangers and ionophores.

A logical property of such small molecule MPACs is to target  $A\beta$  oligomerization and  $A\beta$ -related generation of free radicals: that is to employ MPACs that can prevent reactive metals from participating in potentially harmful redox chemistry. Another important property of a potential MPAC is its ability to cross the BBB. This excludes a large number of common metal chelators as possibilities due to their hydrophilic nature. Nevertheless, there have been two reports of blinded clinical trials of orthodox chelators for the treatment of AD. In 1991, Crapper-McLachlan and colleagues reported benefit over a 2-year period in AD patients treated with intramuscular desferrioxamine twice daily.<sup>116</sup> Desferrioxamine treatment led to significant reduction in the rate of decline of daily living skills, which the authors originally attributed to chelation of aluminum. However, desferrioxamine also chelates zinc, iron and copper. A small double-blind trial of 34 AD subjects with d-penicillamine or placebo reported a decrease in serum oxidative markers over a 6-month period but no change in cognitive decline.<sup>117</sup> However, the large dropout rate in the study led to inconclusive results.



Several metal-complexing agents have been tested for the treatment of AD in a variety of preclinical systems. Derivatives of a 14-membered saturated tetramine, e.g. the bicyclam analogue JKL169 (1,1'-xylyl bis-1,4,8,11 tetraaza cyclotetradecane), have been shown to be effective in reducing copper levels in the cortex and able to maintain normal copper levels in the blood, CSF and corpus callosum of rats.<sup>118</sup> The lipophilic chelator, DP109, has been shown to reduce levels of aggregated insoluble A $\beta$  and conversely increased soluble A $\beta$  forms in a mouse model.<sup>119</sup> In our previous studies, oral treatment with the clioquinol (CQ, 5-chloro-7-iodo-8-hydroxyquinoline) in Tg2576 mice resulted in a reduction of cortical deposition of amyloid (49%) with an improvement or stability in the general health and weight parameters compared to untreated mice.<sup>37</sup> This compound is able to cross the BBB and was able to increase brain copper and zinc levels in treated mice. CQ has nanomolar affinity for Cu<sup>2+</sup> and Zn<sup>2+</sup>,<sup>120</sup> which is sufficient to facilitate dissociation of these metal ions from the low affinity metal binding sites of A $\beta$ , thereby increasing levels of biologically available copper and zinc in the brain of treated animals. Upon peripheral dosing, CQ was demonstrated in Tg2576 mice to cross the BBB and bind to amyloid plaques as well as Zn<sup>2+</sup>-metallated A $\beta$  from post-mortem AD-affected brain specimens.<sup>120</sup> In a small Phase 2 clinical trial, oral administration of CQ in moderately severe AD patients for 36 weeks slowed the rate of cognitive decline and caused a reduction in plasma A $\beta$ 42 levels as compared to the placebo controls.<sup>121</sup> One mode of action of CQ is to strip copper and zinc away from A $\beta$ , thereby preventing oligomerization and promoting the dissolution of non-covalently cross-linked species of A $\beta$ . An alternative mode of action of CQ may be as a modulator of metal levels. CQ appears to have strong ionophore activity and CQ:Cu complexes mediate transport of copper into cells.<sup>122</sup> This results in the activation of matrix metalloproteases and the subsequent degradation of A $\beta$ .<sup>122</sup> Therefore, compounds that target the A $\beta$ :metal interaction and/or metal homeostasis, would appear to have genuine therapeutic potential for treating AD. The goal with this class of MPACs is to remove the copper and zinc from A $\beta$ , and to relocate these metals to sites where they will be beneficial; i.e., to restore metal homeostasis. CQ has also been tested in other neurodegenerative disease models with efficacy shown in both PD<sup>123</sup> and HD<sup>124</sup> animal models. Both of these diseases have been associated with iron overload leading to oxidative stress and free radical generation. CQ was eventually terminated in AD clinical trials due to the generation of a di-iodo contaminant during drug manufacture. More recently, we have found that a second generation 8-OH quinoline derivative of CQ, PBT-2 (Prana Biotechnology, Ltd.), has greater BBB penetration, significantly reduces plaque burden and A $\beta$  levels in transgenic AD mice, improves performance of transgenic AD mice in the Morris Water Maze, rescues A $\beta$ -induced impairment of LTP in hippocampal slices. Following up on these encouraging preclinical findings, Prana Biotechnology recently completed its first double-blind, placebo-controlled Phase 2 clinical trial of 78 AD patients in a 12-week trial for the treatment of mild-moderate AD. An initial report of the results ([http://www.pranabio.com/company\\_profile/press\\_releases\\_item.asp?id=152](http://www.pranabio.com/company_profile/press_releases_item.asp?id=152)) has revealed that the drug was safe and well-tolerated at 50 mg and 250 mg daily doses for 12 weeks, that CSF A $\beta$  levels were significantly lowered at the 250 mg dose for 12 weeks. There was also significant improvement above baseline in performance on two different executive tests (Trail-making B and Verbal Fluency) of the Neuropsychological Test Battery after 12 weeks. These results now serve as the basis for proceeding with further Phase 2b or Phase 3 clinical trials of what may be one of the first disease-modifying drugs based upon the metal theory of Alzheimer's disease.

With regard to mechanism, in mice, CQ is understood to enter the brain and to combine with metallated A $\beta$  in plaques and possibly, soluble pools.<sup>120</sup> CQ treatment of transgenic mice modestly increased brain zinc and copper levels,<sup>37</sup> and in the Phase 2 clinical trial in AD patients the plasma zinc levels significantly increased (normalized from a baseline of deficiency),<sup>121</sup> therefore CQ (and PBT2) do not act as chelators. In cell culture, CQ-Cu complexes enter cells where they markedly inhibit the secretion of A $\beta$  by a mechanism where

the peptide is degraded through up-regulation of matrix metalloprotease (MMP)-2 and MMP-3. MMP activity was increased through activation of phosphoinositol 3-kinase and JNK. CQ-Cu also promoted phosphorylation of glycogen synthase kinase-3, and this potentiated activation of JNK and degradation of A $\beta$ 1-40.<sup>122</sup>

We propose a mechanism of action for treatment of AD where CQ or PBT2 enters the brain and is attracted to the extracellular pool of metals that are in a dissociable equilibrium with A $\beta$ ; e.g., in senile plaques and oligomers (Figure 2). CQ and PBT2 then bind zinc and copper in the A $\beta$  deposits, possibly forming a ternary complex with A $\beta$ . We have previously seen that stripping metals away from A $\beta$  leads to dissolution of A $\beta$  aggregates back down to monomer. A $\beta$  monomer can then be readily cleared from the brain or degraded. Along these lines, an alternative mechanism of action involves the drug-metal complex entering the cell. This then activates MMPs and facilitates the clearance of A $\beta$ ; e.g., in the synapse. In both cases, CQ and PBT2 would also attenuate oxidative cross-linking of A $\beta$  oligomers into covalently bonded species, and reduce the neurotoxic redox activity of A $\beta$  oligomers. Thus, in essence, the MPACS, CQ and PBT2, most likely block A $\beta$  oligomerization and aggregation, dissolve non-cross-linked A $\beta$  aggregates, induce peptidolytic degradation of A $\beta$ , and neutralize A $\beta$  redox activity.

Another interesting approach to metal-based therapeutics for AD targets the increase of iron in the brains of AD patients. This is more a traditional iron chelation therapy, with molecules that pass the BBB and are designed to be multifunctional (by attaching a propargylamine moiety that inhibits acetylcholinesterase), or by exerting antioxidant or monoamine oxidase inhibitor activity.<sup>125-127</sup> By decreasing the labile iron pool, these drugs decrease APP translation (at the 5' UTR iron responsive element, and hence reduce A $\beta$  generation.<sup>128</sup> Iron depletion can also inhibit hypoxia-inducible factor (HIF) prolyl 4-hydroxylases, which have been shown to be neuroprotective.<sup>129</sup> Like all metal complexing agents, it is very difficult to achieve complete metal ion specificity with any structure, and it is likely that these molecules that are thought to target iron will also interact with copper, zinc and other metal ions. This may have advantages in the dissolution of A $\beta$  aggregates, but, as noted above, excess depletion of Cu and Zn may paradoxically exaggerate AD pathology. Only empirical testing can determine whether that will be of value in clinical situation. In any event, it has become increasingly clear that emerging therapies based on the Metal Hypothesis of AD carry considerable promise as a viable therapeutic modality for treating and preventing AD.

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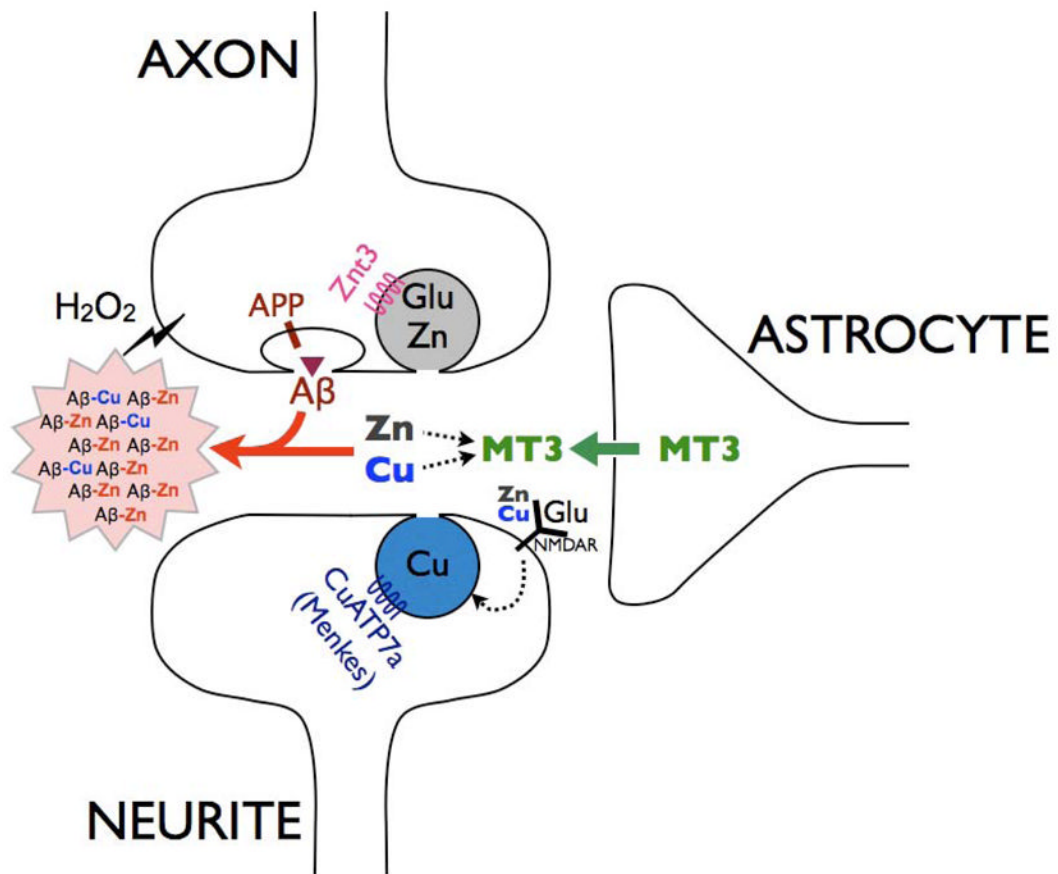
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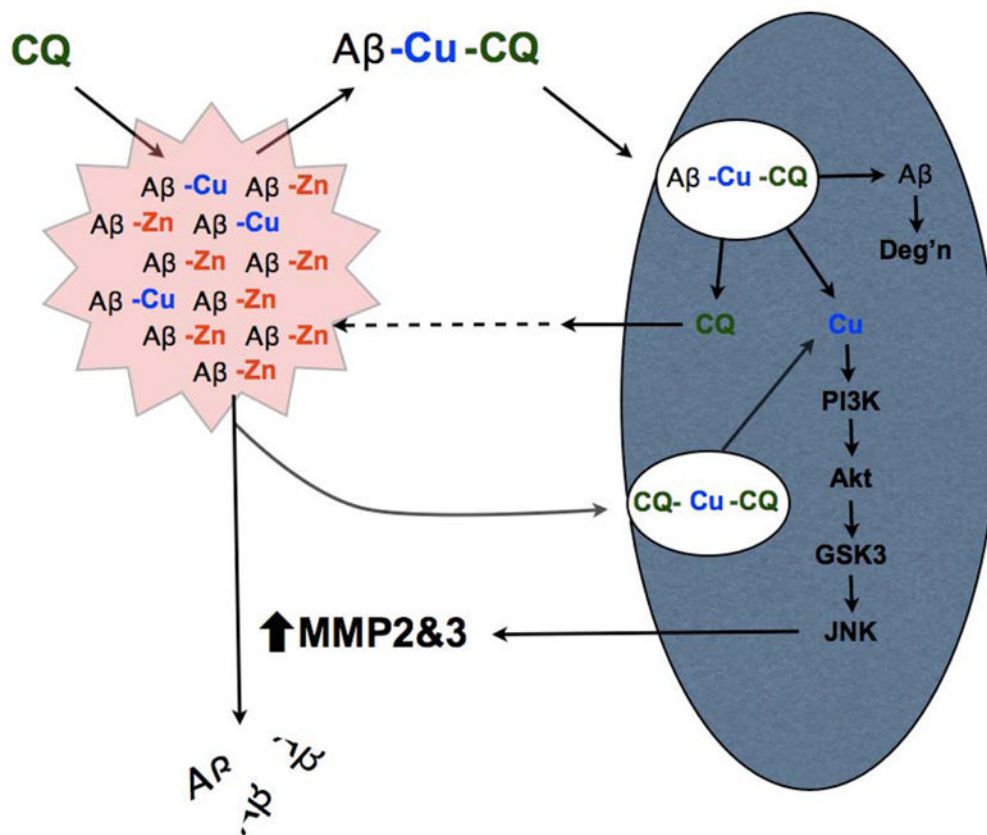
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**Figure 1. Zinc, copper, and Aβ in the glutamatergic synapse**

Zn<sup>2+</sup> is concentrated in the presynaptic bouton by the action of ZnT3, where it may be co-compartmentalized with glutamate, and achieving concentrations up to 300 μM in synaptic clefts. Cu<sup>2+</sup> is released post-synaptically following NMDA-induced activation, which cause the translocation of the Menkes Cu7aATPase and its associated Cu-laden vesicles to the synaptic cleft. Cu<sup>2+</sup> concentrations reach 15 μM in the synaptic cleft. Both Cu and Zn can quench the response of the NMDA receptor. Aβ is released into the synaptic cleft where it has the potential to react with Cu and Zn to form oxidized, cross-linked soluble aggregates and precipitated amyloid. Metallothionein-3 (MT3) released into the cleft by neighboring astrocytes, has the potential to ameliorate this adverse interaction, but is decreased in Alzheimer's disease.



**Figure 2. Mechanism of Action of clioquinol and similar metal-protein attenuation compounds (MPAC)**

The drug enters the brain in the apo-form, where it is first attracted to collections of extracellular metals, a unique feature of AD. The drug combines with the metals (ionic copper or zinc) and facilitates their dissociation (the figure only shows an example for copper and CQ for simplicity, but this generalizes to Zn and other MPACs, like PBT2). The dissociated metal ions may be in a ternary complex with the drug itself or in a complex with dissociated Aβ. These complexes are taken up by neighboring cells, where the elements are separated. The metal ions (copper or zinc) can activate the phosphorylation of GSK-3β, and the activation of MMP2 and 3. This, in turn, facilitates the break down and clearance of Aβ. Not shown are the other predicted benefits of CQ/PBT2, being the dissolution of Aβ aggregates, blocking of cross-linked, covalently bonded Aβ oligomer formation, and the inhibition of toxic Aβ redox activity.



**Table 1**

## Features of Alzheimer's Disease explained by the Metal Theory

| FEATURE   | EXPLANATION  |
|---|--|
| Cortical $\beta$ -amyloid pathology does not occur in rats and mice   | Rat and mouse A $\beta$ have substitutions that attenuate copper and zinc interaction.   |
| $\beta$ -amyloid accumulates primarily in glutamatergic synapses of the neocortex, despite being broadly expressed. | A $\beta$ is precipitated by Zn <sup>2+</sup> and cross-linked by Cu <sup>2+</sup> . ZnT3 is uniquely expressed in neocortical glutamatergic synaptic vesicles, where it functions to concentrate Zn <sup>2+</sup> for release during neurotransmission. Cu <sup>2+</sup> is released from post-synaptic vesicles via an NMDA receptor-mediated mechanism. |
| $\beta$ -amyloid pathology is greater in female transgenic APP mice   | ZnT3 expression is greater in female mice.   |
| $\beta$ -amyloid pathology is age-dependent, even when caused by mutation.  | Brain metal homeostasis fatigues with age.   |